수의학박사학위논문

Application of sonic hedgehog signaling-induced *in vitro* oocyte maturation to *klotho*-knockout pig production

Sonic hedgehog signaling 이 촉진된 체외난자성숙체계의 확립 및 *klotho* 유전자 제거 돼지 생산을 위한 이의 적용

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Application of sonic hedgehog signaling-induced *in vitro* oocyte maturation to *klotho*-knockout pig production

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Department of Veterinary Medicine, Graduate School
Seoul National University

We accept this thesis as confirming to the required standard

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Declaration

This thesis is submitted by the undersigned for examination for the degree of Doctor of Philosophy to the Seoul National University. This thesis has not been submitted for the purposes of obtaining any other degree or qualification from any other academic institution.

I hereby declare that the composition and experiment of this thesis and the work presented in it are entirely my own.

Sanghoon Lee

Application of sonic hedgehog signaling-induced *in vitro* oocyte maturation to *klotho*-knockout pig production

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ABSTRACT

Pigs have been widely used as animal models in biomedical research, because of the similarity of their anatomy and physiology to those of humans. In particular, genetically modified pigs could be used as models for studying human disease and for xenotransplantation by eliminating specific genes. Several techniques for *in*

vitro production of preimplantation porcine embryos have made it possible to produce animals for specific purposes. Among them, somatic cell nuclear transfer (SCNT) is one of the most efficient methods for producing genetically modified pigs. However, the developmental competence of porcine oocytes matured *in vitro*, the most important requirement for SCNT, still remains low. Although numerous studies have been performed to improve *in vitro* maturation (IVM) of porcine oocytes using specific compounds, little consideration has been given to the understanding of physiology of oocyte maturation and underlying mechanism by which they affect porcine cumulus oocyte complexes (COCs).

In growing ovarian follicles, communication among the oocyte, granulosa cell and theca cell compartments regulates each other's proliferation and differentiation via signaling pathways such as sonic hedgehog signaling (Shh), which are essential for follicle development. In addition, targets (Ptch, Smo and Gli1) of active Shh signaling were found in the granulosa and cumulus cell layers of porcine ovary. For these reasons, Shh is considered an important signaling that could affect cumulus expansion and oocyte maturation. Therefore, in this study, the applicability of Shh signaling-induced *in vitro* oocyte maturation to *klotho*-knockout pig production was investigated.

Firstly, the relationship between the beneficial effects of resveratrol or melatonin on porcine IVM and Shh signaling was assessed. To clarify the underlying mechanism by which resveratrol or melatonin directly acts on porcine COCs, cumulus expansion, oocyte nuclear maturation, subsequent embryo development and expression of Shh signaling related genes and proteins were

evaluated. Either a resveratrol or melatonin-induced increase in cumulus expansion, expression of Shh signaling genes, and proteins in cumulus cells and subsequent embryo development was prevented by a Shh signaling inhibitor (cyclopamine). Therefore, it was demonstrated that Shh signaling mediates resveratrol or melatonin to improve cumulus expansion, developmental competence of porcine oocytes and subsequent preimplantation embryo development. Nextly, possible synergistic effects of the combination of resveratrol and melatonin on porcine IVM was investigated. The combination of resveratrol and melatonin has synergistic effects on oocyte nuclear maturation and total cell numbers of parthenogenetic blastocysts and it finally improved cloning efficiency.

Secondly, this improved IVM system was applied to production of *klotho* knockout pigs. The *klotho* gene is considered to be one of the aging-suppressor genes that controls life span. As limited information is available on the functions of the *klotho* gene in large animals such as pigs, *klotho* monoallelic knockout fetal fibroblast cell lines were established by recovery of fetuses cloned via SCNT using non-selected porcine fibroblasts transfected with Cas9-sgRNA ribonucleoproteins, targeting exon 3 of the porcine *klotho* locus. Using these *klotho*-knockout cell lines as nuclear donors, *klotho*-knockout cloned embryos were generated and transferred to eleven recipients to produce *klotho*-knockout pigs. Seven from eleven recipients (63.6%) became pregnant. Nextly, a *klotho* monoallelic knockout cell line inducibly expressing a human *Klotho* was established by transfection of tetracycline (Tet)-inducible vector (pB-Tet-

hKlotho-F2A-*mCherry*) and cloned embryos derived from this cell line were transferred to three recipients. All recipients (100.0%) became pregnant.

In conclusion, Shh signaling mediated the beneficial effects of resveratrol or melatonin on porcine IVM. Furthermore, the combination of resveratrol and melatonin during IVM had synergistic effects on porcine IVM and finally improved cloning efficiency. In addition to this, *klotho* monoallelic fetal fibroblast cell lines with or without inducibly expressing a human *klotho* gene were firstly established in the pig. Using oocytes matured *in vitro* with the combination of resveratrol and melatonin and *klotho*-knockout cell lines as nuclear donors, cloned embryos were generated via SCNT and transferred to recipients to produce *klotho*-knockout pigs. Successful implantation was confirmed through ultrasound imaging 28 days after transfer. Shh signaling-induced IVM system is demonstrated to be applicable in generating genetically modified pigs which are potential animal models for studying human disease.

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Key words: *in vitro* maturation, sonic hedgehog signaling, somatic cell nuclear transfer, *klotho*, pig

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LIST OF ABBREVIATIONS

AC	**Alternating current**
ANOVA	**Analysis of variance**
β-ME	**β-mercaptoethanol**
bp	**Base pair**
BSA	**Bovine serum albumin**
CAG	**Cytomegalovirus enhancer/chicken β-actin**
Cas9	**CRISPR associated protein 9**
cDNA	**Complementary DNA**
CMV	**Cytomegalovirus**
COC	**Cumulus oocyte complex**
CRISPR	**Clustered regularly interspaced short palindromic repeats**
DC	**Direct current**
DMEM	**Dulbecco's modified eagle's medium**
DNA	**Deoxyribonucleic acid**
dNTP	**Deoxynucleotide**
DSB	**Double strand break**
FBS	**Fetal bovine serum**
GAPDH	**Glyeceraldehyde 3-phophate dehydrogenase**
GSH	**Glutathione**
h	**Hour**
Hh	**Hedgehog**

HEPES	**N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]**
hKlotho	**Human klotho**
IGF1	**Insulin-like growth factor 1**
ITS	**Insulin-transferrin-selenium**
IVC	***In vitro* culture**
IVF	***In vitro* fertilization**
IVM	***In vitro* maturation**
kV	**Kilo voltage**
MAPK	**Mitogen-activated protein kinase**
MII	**Metaphase II**
min	**Minute**
MPF	**Maturation promoting factor**
MW	**Molecular weight**
NEAA	**Nonessential amino acid**
PA	**Parthenogenetic activation**
PAM	**Protospacer adjacent motif**
pB	**Piggybac**
PBS	**Phosphate buffered saline**
PCR	**Polymerase chain reaction**
PFF	**Porcine follicular fluid**
P/S	**Penicillin/streptomycin**
PVA	**Polyvinyl alcohol**
PZM	**Porcine zygote medium**

RNA	**Ribonucleic acid**
ROS	**Reactive oxygen species**
RT-PCR	**Reverse transcript-polymerase chain reaction**
SCNT	**Somatic cell nuclear transfer**
sgRNA	**Single-guide ribonucleic acid**
Shh	**Sonic hedgehog**
T7E1	**T7 endonuclease 1**
TALEN	**Transcription activator-like effector nuclease**
TALP	**Tyrode's albumin lactate pyruvate**
TCM-199	**Tissue culture medium-199**
Tet	**Tetracycline**
UV	**Ultraviolet**
ZFN	**Zinc finger nuclease**

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PART I

GENERAL INTRODUCTION

1. Literature review

1.1 Pigs as human disease models

The pig is an excellent biomedical model for studying human disease [1]. Although mice have been widely used to study function of genes [2, 3], there are several limitations in the application of mouse models to translational research. Because of the differences in anatomy, physiology and life span between mice and humans [4], mouse models sometimes cannot reproduce the human disease conditions. To overcome these limitations, the use of large animal models such as pigs in translational research, has gradually increased. In particular, the pig is currently one of the best examples of a large animal model for studying human genetic diseases [5]. Even without genetic modification, pig models have been used for providing an alternative source of organs for xenotransplantation [6] or for studying human infectious diseases [7], wound healing [8], glaucoma [9], cardiovascular disease [10] and diabetes mellitus [11]. In genetically modified models, a number of disease models have been generated over the last decade by targeting specific genomic sites for modification. For example, swine models for human disease, including cystic fibrosis [12], Alzheimer's [13] and Huntington's disease [14], cardiovascular disease [15], breast cancer [16] and Stargardt-like macular dystrophy [17] were created by genetic engineering.

The Swine Genome Sequencing Consortium (SGSC) was formed in September 2003 by academic, government and industry representatives to reveal the pig genome [18]. The pig genome sequence provides an important resource

that enables the acceleration of pig genetics research. In addition to a comparison of pig genomes in multiple breeds, the comparison of pig and human genomes revealed several potential disease-causing genetic variants, which emphasizes the value of pigs in biomedical research.

1.2 Somatic Cell Nuclear Transfer (SCNT) in pigs

SCNT is a laboratory strategy to generate clones of a donor using enucleated oocytes and somatic cells derived from donor animal. Since the first successful cloning of a mammal, a sheep named Dolly in 1997 [19], cloned offspring of many other species (mouse, cattle, goat, pig, rabbit, cat, horse and dog) have been produced via SCNT [20-27]. This technique has received attention because SCNT with genetically modified somatic cells makes it possible to produce genetically modified animals. By introducing and/or removing target genes, these animals can be used for studying the function of genes [28-31].

In 2000, the first and second cloned pigs were reported. Both reports used *in vivo* matured pig oocytes for SCNT and successfully produced cloned piglets. Polejaeva et al. [23] used granulosa cells as a donor cell with a two-stage nuclear transfer technique in which donor cells were fused to enucleated oocytes in the first stage, and the pronucleus-like structures formed were then subsequently transplanted into *in vivo*-produced, enucleated zygotes. In the same year, Onishi et al. [32] also successfully generated cloned pigs using their own approach, a single-step nuclear transfer. Thereafter, cloned piglets were also born using *in vitro* matured oocytes from the ovaries derived from a slaughterhouse [33]. Since the reports of the successful cloned pig production, many researchers have tried to produce genetically modified clones that could be valuable resources for translational research [34-36].

In pig SCNT, the success rate remains low compared to other animals. Usually, pig oocytes and embryos are very sensitive to stressors such as physical changes

generated during *in vitro* embryo production procedures. Pig oocytes contain many lipids inside the cytoplasm [37], which could explain their sensitivity to environmental stressors [38].

Producing cloned pigs via SCNT involves multiple steps, including IVM of oocytes, preparation of donor cells, fusion and activation procedures and *in vitro* culture of embryos, which may influence the results. Among these steps, the IVM condition is the most important and should be optimized to obtain high quality oocytes for successful cloning.

1.3 *In vitro* maturation (IVM) of porcine oocyte

IVM of oocytes can be used both for investigating the physiology of oocyte maturation for basic research purposes and for the large-scale production of mature oocytes for use in subsequent assisted reproductive technologies [39]. Although recent advances in *in vitro* production of preimplantation porcine embryos have enabled us to produce cloned [23] and genetically modified pigs [40], the developmental ability of porcine oocytes produced *in vitro* is still inferior to that of oocytes generated *in vivo*. However, the IVM system has become indispensable for the production of large numbers of competent oocytes in pigs, as it is challenging to obtain *in vivo* matured porcine oocytes consistently [41]. Low developmental competence of porcine oocytes derived from *in vitro* is mainly because of improper IVM culture condition [42]. Oocyte maturation is a critical component of *in vitro* embryo production. If not performed in an appropriate manner under optimal conditions, subsequent embryo development might be compromised [39]. To improve this situation, IVM conditions for porcine oocytes in *in vitro* embryo production systems should be improved [43].

1.3.1 General components of porcine IVM medium

Various basic culture medium types have been used for IVM of pig oocytes, including tissue culture medium (TCM)-199 [44], modified Whitten's medium (mWM) [45], North Carolina State University (NCSU)-23 [46] and -37 [47] and Purdue porcine medium (PPM) for maturation [48].

TCM-199, a common medium for oocyte maturation, has been used in most of the laboratories for porcine oocyte maturation [42, 49-52]. Oocytes matured with TCM-199 have been reported to develop to the blastocysts stage [49, 50, 53] and result in the birth of piglets [49]. However, the blastocyst developmental rate was still low. To overcome this, various supplements were used to improve porcine IVM and subsequent embryo development, as described below.

Epidermal growth factor (EGF) stimulates cell growth and differentiation by binding to its receptor. The presence of mRNA for EGF and its receptor (EGFR) in the oocyte, cumulus and granulosa cells was reported [54], indicating synthesis of EGF and the existence of this signaling in these tissues. Furthermore, the localization of EGF peptide in the oocyte, cumulus, and granulosa cells during all stages of follicles was demonstrated by immunocytochemical techniques. EGF has been shown to stimulate IVM in rats [55], mice [56], cattle [57], and humans [58]. In pigs, EGF can stimulate nuclear maturation, interact with gonadotropins to enhance cytoplasmic maturation [59] and improve subsequent embryo developmental competence [60] .

Supplementation of cysteine in IVM medium increases the amount of glutathione (GSH) in porcine oocytes and improves male pronuclear (MPN) formation after sperm penetration [61] and embryo development [62]. The GSH is one of the endogenous antioxidants in mammalian cells [63], including both gametes [64]. Greater amounts of GSH improve the developmental ability of preimplantation embryos [65].

Pyruvate has a possible bifunctional role as an energy substrate and an antioxidant protecting oocytes against the oxidative stress of the *in vitro* environment [66]. Gamete metabolism is altered during the process of maturation, fertilization and development [67]. Pyruvate metabolism increases during IVM, whereas glucose metabolism decreases [68]. Isolated mouse cumulus cells produced pyruvate when incubated with glucose and lactate [69] and porcine cumulus oocyte complexes (COCs) could produce pyruvate to meet metabolic needs [70]. Although cumulus-denuded oocytes had a limited capacity to use glucose or lactate, they could use pyruvate to support maturation [71]. In addition, developing embryos use pyruvate as their main energy source until the blastocyst stage [72].

ITS (insulin–transferrin–selenium) has been used as an additive to culture medium to partially replace the serum and it is known to promote glucose, amino acid and mineral uptake by cells and to detoxify oxygen radicals [73]. Insulin improved the developmental potential of porcine oocytes during IVM [74]. Transferrin and selenium are integral elements of structure for glutathione peroxidase and are essential for its activity. Thus, they could participate in the antioxidant defense system of the oocytes [75]. ITS has been supplemented to IVM medium in mouse [76], goat [77] and pig [78] and shown beneficial effects.

To improve IVM of oocytes, porcine follicular fluid (PFF) or serum was supplemented to the IVM medium. They contain many unknown factors, such as growth factors and hormones, which can influence the maturation of oocytes [79]. The addition of PFF to IVM medium improved maturation, penetration and

normal fertilization rates [80, 81]. Despite the benefits of PFF, there are some shortcomings, such as the possibility of containing of hidden viruses [82] or variability of effects among sources or batches.

In addition, many studies have investigated the supplementation of various hormones to IVM medium to provide conditions similar to the *in vivo* environment. Effects of follicular stimulating hormone (FSH) [83], luteinizing hormone (LH) [84], estradiol-17β [85], equine chorionic gonadotropin (eCG), human chorionic gonadotropin (hCG) [86] on porcine IVM were investigated and they have shown beneficial effects on IVM. The combination of eCG and hCG was most commonly used for porcine IVM.

In this thesis, I used TCM-199 based IVM medium, as shown in Table 1.

Table 1. Tissue Culture Medium (TCM)-199 maturation medium

Component	Catalog No.	M.W.*	mM
Medium 199 liquid**			
EGF***			10 ng/mL
L-cysteine	C7352	157.62	0.57
Na pyruvate	P4562	110.04	0.91
ITS-A****			10 µl/mL
Kanamycin	K1377	582.58	0.075
PFF*****			10% (v/v)
eCG******	G4877		10 IU/mL
hCG*******	C1063		10 IU/mL

* Molecular weight
** Medium 199, Earle's Salts (Invitrogen; 11150-059, California, USA)
*** Epidermal growth factor (Invitrogen; PHG0314)
**** Insulin-Transferrin-Selenium Solution (ITS-A) (Invitrogen; 51300044)
***** Porcine follicular fluid
****** Equine chorionic gonadotropin
******* Human chorionic gonadotropin

1.3.2 Antioxidants

Despite improvements in porcine IVM over past few decades, obstacles associated with developmental competence of porcine oocyte matured *in vitro* still exist. One of the major problems is improper IVM condition due to oxidative stress caused during *in vitro* culture condition, leading to poor developmental competence of oocytes [87].

Reactive oxygen species (ROS) are intermediary products during a normal process of cellular metabolism [88]. Although ROS are physiologically required for various biochemical pathways, their *in vivo* levels are tightly controlled by an elaborate defense system consisting of enzymatic antioxidants that scavenge and neutralize free radicals [89]. However, *in vitro* environments without the protection of enzymatic antioxidants normally found *in vivo* increase cellular production of ROS, resulting in structural and functional alterations of any molecule by acting as powerful oxidants [90]. These alterations adversely affect developmental competence of oocyte matured *in vitro*. To reduce the oxidative stress, various antioxidants such as resveratrol [91], melatonin [92], cysteine [93], cysteamine [44], ascorbic acid [94], α-tocopherol [95], β-mercaptoethanol [96], canthaxanthin [97] and spermine [98], have been employed to porcine IVM system.

1.3.2.1 Resveratrol

Resveratrol (3,5,4′-trihydroxy-trans-stilbene) is a phytoalexin, generated by several plants such as grapes, plums and peanuts in response to injury or when the

plant is under attack by pathogens such as fungi or bacteria [99]. It exerts a wide variety of pharmacologic activities including anti-inflammatory, antioxidant, antiproliferative, immunomodulative and cardioprotective effects [100-103]. In particular, many studies have been conducted to investigate the effects of resveratrol on IVM of oocytes in several species such as pigs [91, 104], cattle [105, 106] and goats [107]. It was demonstrated that supplementation of 2 μM resveratrol during IVM of porcine oocytes significantly improved oocyte maturation and subsequent embryo development after parthenogenetic activation (PA) [104] and *in vitro* fertilization (IVF) [91] by increasing intracellular GSH and decreasing ROS levels. In cattle, resveratrol treatment of 1 μM during IVM significantly improved cumulus expansion, polar body formation, the (hatched) blastocyst rate and the mean number of cells/blastocysts by inducing progesterone secretion and an antioxidant effect, probably in a manner dependent on sirtuin-1 [106]. Another group [105] examined the effects of supplementation of resveratrol on the expression levels of SIRT1 in oocytes and found that 20 μM resveratrol significantly increased the expression of SIRT1 and conducted further experiments using this concentration. They demonstrated that resveratrol improved the quality of oocytes by improving mitochondrial quantity and quality. In goats [107], oocytes treated with 0.25 and 0.5 μM resveratrol showed significantly higher extent of blastocyst yields after PA and handmade cloning by increasing the intracellular GSH and decreasing ROS levels.

There was a difference in the optimal concentration of resveratrol for IVM among species. This might be due to different culture period and/or physiology of

maturation among species. Most studies explain the beneficial effects of resveratrol mainly by indirect antioxidant effects. As resveratrol has various biological activities, there would be more direct actions of resveratrol on porcine COCs. Therefore, it is required to investigate the underlying mechanism by which resveratrol directly acts on porcine COCs.

1.3.2.2 Melatonin

Melatonin, also known as *N*-acetyl-5-methoxytryptamine, a hormone that is produced by the mammalian pineal gland and regulates sleep and wakefulness [108]. Various biological activities of melatonin were well studied, including antioxidant [109], immune defense [110], anti-inflammatory [111] and anti-cancer effects [112].

Several evidences suggest a local role of melatonin in reproductive processes. For example, melatonin influences ovarian function directly [113] and the presence of melatonin receptors in cells of the ovary [114, 115] and melatonin in ovarian follicular fluid [116] were reported. Based on these backgrounds, numerous studies have been performed to investigate the effects of melatonin on IVM of oocytes in mice [117], humans [118], cattle [119-122] and pigs [92, 123-125]. In mice, melatonin in the concentration of 10^{-6} M significantly enhanced maturation of oocytes [117]. In humans, 10^{-9} M of exogenous melatonin treatment during IVM showed significantly higher nuclear maturation of oocytes [118]. In cattle, melatonin supplementation at 10 and 50 ng/ml to IVM medium significantly increased oocyte nuclear maturation, cumulus cell expansion and

altered the mitochondrial distribution patterns with decreased intracellular ROS levels. Other groups reported that 10^{-9} M melatonin significantly increased maturation of oocytes and subsequent embryo development [120, 121]. In pigs, Shi et al. [123] demonstrated that melatonin exists in PFF and exogenous melatonin at 10^{-9} M during IVM improved subsequent embryo development after PA. Jin et al. [125] also reported that 10^{-9} M melatonin treatment during IVM significantly increased embryo development after PA.

Most studies explained that the beneficial effects of melatonin on IVM were due to its indirect antioxidant effects. However, because many of its biological effects are mediated through activation of melatonin receptors [126] or its indirect antioxidant effects [109], there might be a possible direct action of melatonin on porcine COCs through melatonin receptors. Therefore, it is necessary to investigate a potential mechanism underlying the beneficial effects of melatonin on porcine COCs.

1.4 Sonic hedgehog signaling

The Hedgehog (Hh) signaling pathway was first identified in Drosophila melanogaster [127] and it is now known as one of the key regulators of animal development, such as gonad development and many other events during the course of development [128, 129]. In vertebrates, there are three hedgehog homologues, namely Sonic Hh (Shh), Indian Hh (Ihh) and Desert Hh (Dhh), of which Sonic is the best studied [130-132]. The pathway is equally important during vertebrate embryonic development and is therefore of interest in developmental biology. The signaling of the Hh family peptides require the mediation by a cell surface receptor system consisting of two proteins. When Shh reaches its target cell, it binds to the Patched-1 (Ptch1) receptor. In the absence of ligand, Ptch1 represses Smoothened (Smo), a downstream protein in the pathway [133-135]. Whereas the binding of Shh to Ptch1 relieves the inhibition of Smo, leading to activation of the Gli transcription factors: the activators Gli1 and Gli2 and the repressor Gli3 [136]. Activated Gli accumulates in the nucleus and controls the transcription of Hh target genes.

1.4.1. General understanding of the *in vivo* physiology of oocyte maturation

Oocyte development in the mammalian ovary requires productive interactions with somatic granulosa cells of the ovarian follicle [137]. Granulosa cells participate in the processes of follicular proliferation, differentiation, ovulation, lutenization and atresia. Particularly, proliferating granulosa cells support the progression of follicular growth and oocyte maturation. Many ovarian factors are

involved in the regulation of this process via different molecular mechanisms and signal pathways [138].

Previous studies have investigated Hh signaling pathways in developing and adult mouse ovaries. Ihh and Dhh are expressed in the granulosa cells of preantral and antral follicles, with their receptors Ptch1 and Smo expressed in thecal cells. These results suggest that paracrine signaling communication through Hh may exist between granulosa and theca cells [130, 136]. Furthermore, Russell et al. [132] reported that three Hh ligands (Shh, Ihh and Dhh) and their receptors (Ptch1 and Smo) are expressed in immature and adult mouse ovaries. Furthermore, they revealed that when preantral follicles were supplemented with Shh *in vitro*, the proliferation of granulosa cells was increased. However, concomitant treatment with Shh and its inhibitor (cyclopamine) blocked Shh-induced follicular growth. In pigs, Shh and its downstream molecules (Ptch1, Smo and Gli1) are expressed in the ovary. Expression of Ptch1 and Smo was detected, particularly in the granulosa cells surrounding the oocyte and the oocyte itself [139]. Based on these results, the authors concluded that the Hh signaling pathway may be involved in granulosa cell proliferation and oocyte maturation.

1.5 *Klotho*

The *klotho* gene encodes a single-pass transmembrane protein that is related to β-glucuronidases. This gene is mainly expressed in distal convoluted tubules in the kidney and choroid plexus in the brain [140]. Mutations within this gene in mice (the *klotho* mice) leads to a syndrome resembling aging such as growth retardation, infertility, arteriosclerosis, ectopic calcification in various soft tissues, osteoporosis, skin atrophy, muscle atrophy, pulmonary emphysema and finally premature death at 2 months of age [141]. Whereas, transgenic mice that overexpress *klotho* showed extended life span by 20% [142]. Later, klotho deficient mice ($KL^{-/-}$) were generated by conventional gene targeting and exhibited aging-like phenotypes identical with those observed in the original *klotho* mice [143]. Thus, the *klotho* gene was identified as an aging suppressor gene, extending life span when overexpressed and accelerating aging when inactivated [144].

Although klotho protein is predominantly expressed in the kidney, it is also detected in other tissues, such as the placenta, ovary, prostate gland and small intestine [141, 145]. The klotho protein is composed of two domains; a large extracellular domain (130 kDa), a transmembrane domain and a very short intracellular domain (10 amino acids). The extracellular domain contains homology to family 1 glycosidases (enzymes that hydrolyze terminal glycosidic linkages in glycoproteins and glycolipids) and is subject to ectodomain shedding, being released into the extracellular space such as blood, urine and cerebrospinal fluid [142, 146-148]. Therefore, the klotho protein exists in two forms: membrane

and secreted forms. Membrane klotho functions as a receptor for a hormone that controls excretion of phosphate and synthesis of vitamin D in the kidney [149-151]. Secreted klotho functions as a humoral factor that inhibits intracellular signaling cascades, resulting in suppression of oxidative stress [152].

Many studies have been conducted to reveal functions of this protein and recently, the klotho protein is defined as a multi-functional protein involved in calcium and phosphate homeostasis [153], the insulin/insulin-like growth factor 1 (IGF1) signal pathway [154], apoptosis [155], angiotensin II-induced events in the kidney [156] and oxidative stress [152]. However, it is still unclear what mechanisms are involved in the aging suppression effects of klotho.

Producing cloned pigs via SCNT involves multiple steps, including IVM of oocytes, preparation of donor cells, fusion and activation procedures and *in vitro* culture of embryos, which may influence the results. Among these steps, the IVM conditions are most important and should be optimized to obtain high quality oocytes for successful cloning.

2. General objective

The purpose of this study is to establish Shh signaling-induced *in vitro* oocyte maturation system and apply this system to *klotho*-knockout pig production. This thesis is composed of 5 parts. In part I; as a general introduction, background and experimental design of this study were explained. In part II; general methodology used in this study was described. In part III; the relationship between Shh signaling pathway and beneficial effects of 1) resveratrol or 2) melatonin on porcine IVM was investigated. Finally, 3) possible synergistic effects of resveratrol and melatonin were investigated to find the optimal condition for production of cloned pig. In part IV; 1) *klotho*-knockout cell lines and 2) *klotho*-knockout cell lines inducibly expressing a human *klotho* gene were established and cloned embryos using these cell lines were transferred to synchronized recipients for production of *klotho*-knockout pigs using Shh signaling-induced IVM system. In part V; a final conclusion of this study was described.

PART II

GENERAL METHODOLOGY

1. Chemicals and materials

All chemicals and reagents used in this study were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA), unless otherwise stated.

2. *In vitro* maturation

Porcine ovaries were collected from prepubertal gilts at a local abattoir and transported to the laboratory within 3 h in physiological saline at 30-32 °C. Cumulus oocyte complexes (COCs) were aspirated from 3 to 6 mm diameter superficial ovarian follicles using an 18-gauge needle attached to a 10 mL disposable syringe and allowed to sediment in 50 mL conical tubes at 37 °C for 5 min. The supernatant was discarded and the sediment was washed three times in washing medium containing 9.5 g/l of TCM-199 (Invitrogen, Carlsbad, CA, USA), 5 mM sodium hydroxide, 2 mM sodium bicarbonate, 10 mM N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES), 0.3% polyvinyl alcohol (PVA), and 1% Pen-Strep (Invitrogen). Subsequently, COCs were recovered while observing under a stereomicroscope. Only COCs with ≥3 uniform layers of compact cumulus cells and a homogeneous oocyte cytoplasm were selected, and washed three times in washing medium. Approximately 50 COCs were transferred to IVM medium comprising TCM-199 supplemented with 10 ng/mL epidermal growth factor, 0.57 mM cysteine, 0.91 mM sodium pyruvate, 5 μL/mL ITS-A 100X (Invitrogen), 10% porcine follicular fluid, 10 IU/mL eCG, and 10 IU/mL hCG. The selected COCs were incubated at 39 °C under 5% CO_2 in 95%

humidified air for IVM. After 21-22 h of maturation culture with hormones, the COCs were washed twice in fresh hormone-free IVM medium and then cultured in hormone-free IVM medium for an additional 21-22 h.

3. General cell culture

3.1. Primary culture of porcine fetal fibroblasts

The euthanized fetus was dissected into three parts: head, body, and tail. Just the body part of fetus was washed three times in phosphate-buffered saline (PBS; Gibco, Carlsbad, CA, USA) containing 1% Penicillin/Streptomycin (P/S; Gibco) and then chopped into small pieces in a 60-mm dish with Dulbecco's Modified Eagle's Medium (DMEM; Gibco). Well-dissociated tissues were centrifuged at 1,500 rpm for 2 min. The supernatant was discarded, and the pellet was resuspended with DMEM and then centrifuged at 1,500 rpm for 2 min. These procedures were repeated two times. Finally, the supernatant was discarded, and the pellet was resuspended in DMEM supplemented with 20% fetal bovine serum (FBS, Gibco), 1% P/S, 1% nonessential amino acid (NEAA; Gibco) and 100 mM β-mercaptoethanol (β-ME) by inverting the tube several times. The suspension was transferred to a cell culture dish for ~10 days and the culture medium was changed every 2–3 days. These primary cells were cultured, expanded and frozen at −196 °C for further use. The cell cultures were maintained in DMEM with 20% FBS, 1% P/S, 1% NEAA and 100 mM β-ME.

3.2. Cell culture of porcine fibroblasts

Porcine fibroblasts were placed in a tissue-culture dish containing general cell culture medium: DMEM supplemented with 20% FBS and 1% P/S, 1% NEAA and 100 mM β-ME. Expanded cells were maintained in cell culture medium at 39 °C under 5% CO_2 in 95% humidified air. During cell proliferation on the same dish, the culture medium was changed every day. For subculture or cryopreservation, cultured porcine cells were treated with 0.25 % Trypsin-EDTA (Gibco) for 2 min in an incubator at 37 °C, when the cell population reached about 80-90 % of culture dishes. The digested cells were collected by centrifugation at 1,500 rpm for 2 min. After the supernatant was discarded, the cell pellet was suspended with culture medium and subcultured to a new tissue-culture dish with optimized cell numbers. Cryopreservation was performed using cryopreservation medium containing 70% culture medium, 20% FBS and 10% dimethyl sulfoxide and the cryopreserved cells were stored at -196 ℃.

4. Preparation of somatic cell nuclear transfer in pig

4.1. Donor cell preparation

Two days before the SCNT, the donor cells were prepared by sub-culturing or thawing in the 4-well cell culture dish with 500 μl cell culture medium. After two days of culture, prepared donor cells were treated with 0.25% Trypsin-EDTA for detachment from the dish in 37 °C incubator for 2 min. The digested cells were collected by brief centrifugation and the supernatant was discarded. Cells were washed with PBS and collected again by brief centrifugation. Finally, the cell pellet was resuspended with Tyrode's albumin lactate pyruvate (TALP) medium

and stored in room temperature before injection.

4.2. Somatic cell nuclear transfer and embryo culture

After 40 hours of IVM, denuded oocytes were stained with 5 μg/mL of bisbenzimide (Hoechst 33342) for 10 min and observed under an inverted microscope equipped with epifluorescence. An oocyte was held with a holding micropipette, and the zona pellucida was partially dissected with a fine glass needle to make a slit near the first polar body. Enucleation was performed by aspirating the first polar body and adjacent cytoplasm containing the metaphase-II chromosomes with an aspiration pipette in TALP medium containing 7.5 μg/mL of cytochalasin B. After enucleation, using a fine pipette, a trypsinized porcine fetal fibroblast with a smooth cell surface was transferred into the perivitelline space of an enucleated oocyte. These couplets were equilibrated with fusion solution (0.26 M mannitol solution containing 0.5 mM HEPES and $MgSO_4$) for 4 min and then fused in a 20 μL droplet of fusion solution with a single direct current (DC) pulse of 1.2 kV/cm for 30 μs using an electrical pulsing machine (LF101; Nepa Gene, Chiba, Japan). After 30 min, fused couplets were equilibrated with activation solution (0.28 M mannitol solution containing 0.5 mM HEPES, 0.1 mM $CaCl_2$ and $MgSO_4$) for 4 min, transferred to a chamber containing two electrodes overlaid with activation solution, and activated with a single DC pulse of 1.5 kV/cm for 30 μs using a BTX Electro-Cell Manipulator 2001 (BTX Inc., San Diego, CA, USA). The resulting activated embryos were washed three times with fresh Porcine Zygote Medium (PZM-5; Funakoshi Corporation, Japan) and

cultured in PZM-5 at 39 °C in a humidified atmosphere of 5% O_2, 5% CO_2, and 90% N_2 for 7 days.

5. Embryo transfer and pregnancy test

Two days after standing estrus was observed, the surrogate pig was restrained and preanesthesia was induced by injection of ketamine (10 mg/kg; Yuhan, Seoul, Korea) and xylazine (1mg/kg; SF Inc., Ansan, Korea) into a vein in the ear. The anesthetized surrogate was placed on a surgery table in a ventrodorsal posture and general anesthesia was maintained by isoflurane with concentration of 2.0 MAC (Hana Pharm., Seoul, Korea). The reproductive tract was taken out from abdomen through a midline incision and exposed. The SCNT embryos were loaded into a 3.5 Fr Tomcat catheter (Covidin, Dublin, Ireland) with TALP medium and transferred into both oviducts of the recipient pig through a small puncture made with a suture needle (Covidin). The empty Tomcat catheter was removed carefully, the uterus and ovaries were put into the abdominal cavity and the opened abdomen was sutured. Pregnancy was diagnosed by ultrasonography on Day 25 (The day of SCNT was considered Day 0). If the surrogate became pregnant, the gestation was monitored every two weeks.

6. Statistical analysis

Statistical analyses were performed using SPSS 22.0 (SPSS, Inc., Chicago, IL, USA). All data were tested for normality and homoscedasticity. The Mann-Whitney U test (for data with non-normal distribution) or Student's *t*-test (for data

with normal distribution) was used to determine differences between two groups. The Kruskal-Wallis test (for data with non-normal distribution) or one-way analysis of variance (ANOVA) (for data with normal distribution) was used to determine differences among three or more groups. The one-way ANOVA test was followed by Duncan's multiple range test (when the variances were assumed to be equal) or Dunnett's T3 test (when the variances were assumed to be unequal). All results are expressed as means ± SEM. P values < 0.05 were considered to be statistically significant.

PART III

THE IMPROVEMENT OF *IN VITRO* MATURATION SYSTEM FOR *KLOTHO*-KNOCKOUT PIG PRODUCTION

Chapter I. Sonic hedgehog signaling mediates resveratrol to improve maturation of pig oocytes *in vitro* and subsequent preimplantation embryo development

1. Introduction

Resveratrol (trans-3,5,4'-trihydroxystilbene) is a polyphenolic phytoalexin, a secondary plant metabolite, generated by the interactions between plants and a microorganism found in grapes, plums, peanuts and mulberries [157]. Previous studies have reported that resveratrol has anti-oxidant, anti-cancer, anti-inflammatory and anti-aging properties [100, 102, 158]. In addition, it has been demonstrated that resveratrol improves IVM of oocytes and subsequent embryo development in various species including pigs [91], cattle [105, 106] and goats [107]. I also have previously reported that 2 μM resveratrol improves IVM of porcine oocytes and their subsequent embryo development [104]. However, these beneficial effects of resveratrol on IVM have been explained mainly by indirect antioxidant effects and mechanisms underlying direct actions of resveratrol have not been well studied. Therefore, the present study was focused on the ways in which resveratrol directly acts on porcine COCs.

Shh is a crucial signaling pathway functioning in an autocrine and/or paracrine fashion [159], which enhances cell proliferation and differentiation in many cell types [160, 161]. Shh exerts its action by binding to its receptor, Ptch1 on the cell surface, which leads to Ptch1 internalization and degradation, resulting in the inhibition of Smo, the seven-transmembrane G-protein-coupled coreceptor.

Suppressed Smo then permits nuclear translocation of the Gli1 transcription factor that regulates cell patterning, proliferation, migration and differentiation during development [162]. Recently, several studies reported that Shh signaling mediates resveratrol to exert its biological activities. This signaling mediates resveratrol to increase neural stem cell proliferation after oxygen-glucose deprivation/reoxygenation injury *in vitro* [163] and also mediates resveratrol to decrease cerebral ischemic injury and improve neurological function after strokes [164]. These results indicated that Shh signaling could be a candidate pathway for resveratrol-induced improvement in oocyte maturation.

Furthermore, interactions between the oocyte and surrounding somatic cells in growing ovarian follicles regulate each other's proliferation and differentiation [165] via signaling pathways such as Shh, which modulates follicle development [132]. Recently, the existences of active Shh signaling targets (Ptch, Smo and Gli1) were found in the granulosa and cumulus cell layers of the ovary in various species including mice [136], cattle [166] and pigs [139]. Furthermore, addition of recombinant Shh protein during porcine IVM improved oocyte maturation and subsequent *in vitro* development [139, 167].

Previous researches implied that the Shh signaling pathway might be a promising target of resveratrol involved in its beneficial effects on oocyte maturation. However, the role of the Shh signaling pathway in the effects of resveratrol on porcine IVM has not been investigated. Therefore, the aim of this study was to determine the relationship between the beneficial effects of resveratrol on porcine IVM and Shh signaling. In this study, Shh signaling was

inhibited using cyclopamine, an inhibitor of Smo (one of the Shh signaling molecules) [168], to confirm whether Shh signaling is induced by resveratrol. I investigated the effects of resveratrol with or without cyclopamine during IVM on cumulus expansion, oocyte nuclear maturation, subsequent embryonic development and expression of genes and proteins related to Shh signaling.

2. Materials and methods

2.1. Oocyte recovery and IVM

Procedures for IVM were described in general methodology.

2.2 Cumulus expansion assessment

The degree of cumulus expansion was assessed by microscopic examination as described previously [169]. In brief, a degree of 0 indicates no detectable expansion, characterized by detachment of cumulus cells from the oocyte leaving a partially or fully denuded oocyte. A degree of 1 indicates the minimum observable response with spherical and compacted cumulus cells around the oocyte. A degree of 2 indicates only the outermost layers of cumulus cells have expanded. A degree of 3 indicates all cell layers except the corona radiata expanded and a degree of 4 indicates the maximum degree of expansion including the corona radiata.

2.3. Assessment of nuclear maturation

After 42–44 h of IVM, COCs were denuded by gently pipetting with 0.1% hyaluronidase in TALP medium and washed three times in TALP medium. The denuded oocytes were evaluated under a microscope (TE2000-S, Nikon Corp., Tokyo, Japan) and classified as immature (without first polar body extrusion), degenerate, or at metaphase II (with polar body extrusion).

2.4. Parthenogenetic activation of oocytes

Denuded oocytes were gradually equilibrated in activation medium consisting of 0.28 M mannitol, 0.5 mM HEPES, 0.1 mM $CaCl_2$ and 0.1 mM $MgSO_4$ and transferred into a chamber connected to a BTX Electro-Cell Manipulator 2001 (BTX Inc.). Oocytes were activated by a single DC pulse of 1.5 kV/cm for 60 μs. Then, electrically-activated oocytes were washed three times in fresh PZM-5 (Funakoshi Corporation), transferred into 500 μL PZM-5 wells and cultured at 39 °C in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2 for 7 days.

2.5. Embryo evaluation and total cell count after PA

The day of PA was considered Day 0. Cleavage was evaluated on Day 2 (48 h) and blastocyst formation was assessed on Day 7 (168 h). To count the total cell numbers of blastocysts, Day 7 blastocysts were washed in TALP medium and then stained with 5 μg/mL bisbenzimide (Hoechst-33342) for 10 min. After a final wash in TALP medium, stained blastocysts were mounted on glass slides in a drop of 100% glycerol, gently flattened with a cover glass, and observed under a fluorescence microscope (Nikon Corp.) at 400× magnification.

2.6. Gene expression analysis by real-time PCR

Total RNA was extracted from cumulus cells, oocytes and PA-derived blastocysts using the easy-spin Total RNA Extraction Kit (iNtRON, Seoul, Korea) according to the manufacturer's protocol. The eluted total RNAs were quantified by a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington,

DE, USA). Complementary DNA (cDNA) was synthesized using the RNA to cDNA EcoDry Premix, cDNA synthesis kit (Clontech Laboratories Inc., Mountain View, CA, USA) according to the manufacturer's protocol. The following were placed in a MicroAmp optical 96-well reaction plate (Applied Biosystems, Singapore, Singapore): 1 μL cDNA (2.5-25 ng/μL), 0.4 μL (10 pmol/μL) forward primer, 0.4 μL (10 pmol/μL) reverse primer, 10 μL SYBR Premix Ex Taq (Takara, Otsu, Japan), and 8.2 μL of Nuclease-free water (Ambion, Austin, TX, USA). The reactions were carried out for 40 cycles and the cycling parameters were as follows: denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min. Primer sequences, expected product sizes, and GenBank accession numbers for the real-time PCR analysis are presented in Table 2. The expression of each target gene was quantified relative to that of the internal control gene (*GAPDH*) using the equation, $R = 2^{-[\Delta Ct\ sample - \Delta Ct\ control]}$. Each experiment was replicated at least three times.

2.7. Immunocytochemical staining

In vitro matured COCs were washed three times in PBS containing 0.2% PVA and fixed with 4% paraformaldehyde (w/v) in PBS for 1 h at room temperature. After washing three times in PBS with 0.2% PVA, COCs were treated with 1% (v/v) Triton X-100 in PBS for 30 min at 37 °C for permeabilization. The COCs were washed three times in PBS with 0.2% PVA and blocked with 2% bovine serum albumin (BSA) in PBS overnight at 4 °C. Subsequently, the COCs were incubated with primary antibodies for Shh (1:200; sc-365112; Santa Cruz

Biotechnology, Inc., Santa Cruz, CA, USA), Ptch1 (1:200; sc-9016; Santa Cruz), Smo (1:200; sc-13943; Santa Cruz) or Gli1 (1:200; sc-20687; Santa Cruz) at 37 °C for 3 h. After washing three times in PBS with 2% BSA, the COCs were reacted with the appropriate secondary antibodies as follows: anti-mouse monoclonal antibody (1:200; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) for Shh or anti-rabbit polyclonal antibody (1:200; ab6717, Abcam, Cambridge, UK) for Ptch1, Smo and Gli1 at 37 °C for 1 h (in darkness). Samples were finally washed three times in PBS with 2% BSA and mounted on glass slides. Images were captured with a confocal laser-scanning microscope (SP8 X, Leica, Mannheim, Germany). The intensities of Shh, Ptch1, Smo and Gli1 were measured by Image J software (version 1.46r; National Institutes of Health, MD, USA).

2.8. Experimental design

To elucidate the relationship between resveratrol and Shh signaling, I designed three groups: (1) control, (2) resveratrol, and (3) resveratrol with cyclopamine (Shh signaling inhibitor). They were added to the IVM medium during the entire maturation period of 44 h. The concentrations of resveratrol (2 μM) [104] and cyclopamine (2 μM) [139, 170] were set according to these previous studies. In experiment 1, to exclude the possibility that reduction in resveratrol-induced improvements can occur by inhibition of endogenous Shh signaling by cyclopamine, I investigated the effects of cyclopamine on cumulus expansion, oocyte nuclear maturation, subsequent embryo development and expression of

genes related to Shh signaling in cumulus cells. In experiment 2, I investigated the degree of cumulus expansion, expression of genes related to cumulus expansion and Shh signaling and expression of proteins related to Shh signaling in cumulus cells. In experiment 3, I evaluated the oocyte maturation rate, expression of genes related to oocyte competence, maturation promoting factor (MPF), the mitogen-activated protein kinases (MAPK) pathway and Shh signaling and expression of proteins related to Shh signaling in oocytes. In experiment 4, I assessed *in vitro* development of PA embryos and expression of genes related to developmental competence, glucose metabolism and apoptosis in PA blastocysts.

2.9. Statistical analysis

Procedures for statistical analysis were described in general methodology.

Table 2. List of real-time PCR primers

Gene	Primer sequences (5'-3')		Product size (bp)	GenBank accession number
	Forward	Reverse		
GAPDH	GTCGGTTGTGGATCTGACCT	TTGACGAAGTGGTCGTTGAG	207	NM_001206359
Shh	ACTGACCCCTTTAGCCTACA	GTCGGCTCCAGTGTTTTCTT	172	NM_001244513
Ptch1	CTCGGGAAACGAGAGAATAC	AGTAGTGCAGCCACATTTTG	198	XM_003482800
Smo	AGAGATACGTGCTCGCTCTT	CTGTCTGGTGCTTGAAACTG	201	XM_003134680
Gli1	AGAGGGACAGCTCTGAACAC	GCTACGTCTCTTCCTCCTGA	199	NM_001256593
Ptgs1	CAACACGGCACACGACTACA	CTGCTTCTTCCCTTTGGTCC	121	XM_001926129
Ptgs2	ACAGGGCCATGGGGTGGACT	CCACGGCAAAGCGGAGGTGT	194	NM_214321
Has2	AGTTTATGGGCAGCCAATGTAGTT	GCACTTGGACCGAGCTGTGT	101	AB050389
Ptx3	GGCCAGGGATGAATTTTAC	GCTATCCTCTCCAACAAGTGA	185	NM_001244783
Tnfapi6	AGAAGCGAAAGATGGGATGCT	CATTTGGGAAGCCTGGAGATT	106	NM_001159607
Gdf9	CAGTCAGCTGAAGTGGGACA	TGGATGATGTTCTGCACCAT	135	AY626786
Bmp15	CCTCCATCCTTTCCAAGTCA	GTGTAGTACCCGAGGGCAGA	112	NM_001005155
Cyclinb1	CAACTGGTTGGTGTCACTGC	TTCCATCTGCCTGATTTGGT	126	L48205
Cdc2	GGGCACTCCCAATAATGAAGT	GTTCTTGATACAACGTGTGGGAA	260	AB045783
C-mos	GGGAGCAACTGAACTTGGAG	AGAATGTTCGCTGGCTTCAG	115	NM_001113219
Pou5f1	TTTGGGAAGGTGTTCAGCCAAACG	TCGGTTCTCGATACTTGTCCGCTT	198	NM_001113060

Sox2	ATGCACAACTCGGAGATCAG	TATAATCCGGGTGCTCCTTC	130	NM_001123197
Nanog	GGTTTATGGGCCTGAAGAAA	GATCCATGGAGGAAGGAAGA	98	NM_001129971
Glut1	GCTTCCAGTATGTGGAGCAA	AAGCAATCTCATCGAAGGTC	132	XM_013977359
Bax	TGCCTCAGGATGCATCTACC	AAGTAGAAAAGCGCGACCAC	199	XM_003127290
Bcl2	AGGGCATTCAGTGACCTGAC	CGATCCGACTCACCAATACC	193	NM_214285

3. Results

3.1. Effects of Shh signaling inhibitor on cumulus expansion, oocyte nuclear maturation, subsequent embryo development and Shh signaling in cumulus cells

In the first experiment, the effects of 2 μM cyclopamine treatment during IVM on cumulus expansion, oocyte nuclear maturation, subsequent embryo development and expression of Shh signaling genes in cumulus cells were investigated (Fig. 1A-1D). Cyclopamine significantly decreased the expression of SHH signaling genes (*Shh*, *Ptch1*, *Smo* and *Gli1*). However, there were no significant differences in cumulus expansion, oocyte nuclear maturation and subsequent embryo development.

3.2. Effects of resveratrol with or without Shh signaling inhibitor on cumulus expansion and Shh signaling in cumulus cells

In experiment 2, I investigated the effects of 2 μM resveratrol with or without 2 μM cyclopamine treatment during IVM on cumulus expansion, expression of genes related to cumulus expansion (*Ptgs1*, *Ptgs2*, *Has2*, *Ptx3* and *Tnfaip6*) and expression of Shh signaling genes (*Shh*, *Ptch1*, *Smo* and *Gli1*) and proteins (Shh, Ptch1, Smo and Gli1). Resveratrol significantly increased the proportion of COCs exhibiting complete cumulus expansion (degree 4) and decreased the proportion of degree 1 compared to the control (Fig. 2). However, these effects were blocked by simultaneous addition of cyclopamine. At the same time, increased expression of genes related to cumulus expansion (*Ptgs1*, *Ptgs2*, *Has2*, *Ptx3* and *Tnfaip6*) after treatment with resveratrol was also inhibited by

cyclopamine (Fig. 3A). To further investigate the relationship between the effect of resveratrol on cumulus expansion and Shh signaling, the expression of Shh signaling genes and proteins was examined in cumulus cells. Resveratrol showed significantly increased expression of Shh signaling genes (*Shh*, *Ptch1*, *Smo* and *Gli1*) and proteins (Ptch1, Smo and Gli1). However, cyclopamine depressed these resveratrol-induced increases in expression of Shh signaling genes and proteins (Fig. 3B and 6B). Overall, expression patterns of Shh signaling genes and proteins, which were increased by resveratrol and blocked in the presence of cyclopamine, were similar to those observed from the proportion of COCs exhibiting complete cumulus expansion (degree 4) and expression of cumulus expansion genes (*Ptgs1*, *Ptgs2*, *Has2*, *Ptx3* and *Tnfaip6*).

3.3. Effects of resveratrol with or without Shh signaling inhibitor on oocyte nuclear maturation and Shh signaling in oocytes

In experiment 3, the effect of 2 μM resveratrol with or without 2 μM cyclopamine treatment during IVM on oocyte nuclear maturation was investigated. As shown in Figure 4, resveratrol significantly increased metaphase II rates and decreased immature and degeneration rates compared to the control (94.3%, 2.7% and 3.0% vs. 88.3%, 5.0% and 6.7%, respectively). However, the combined cyclopamine and resveratrol group showed no significant differences in metaphase II, immature and degeneration rates (90.4%, 3.9% and 5.7%) compared to the control. Then, I further examined the expression of genes related to oocyte competence (*Gdf9* and *Bmp15*), MPF (*Cyclinb1* and *Cdc2*), the MAPK pathway (*C-mos*) and expression of Shh signaling genes and

proteins in oocytes. Expression of an oocyte competence gene (*Gdf9*), MPF genes (*Cyclinb1* and *Cdc2*) and a MAPK pathway gene (*C-mos*) was significantly increased by resveratrol treatment, but the effect was counteracted by cyclopamine (Fig. 5A-5C). Moreover, resveratrol significantly increased expression of Shh signaling genes (*Shh*, *Ptch1*, *Smo* and *Gli1*) and proteins (Ptch1, Smo and Gli1) in oocytes and again cyclopamine abolished this increase except for a Shh gene and a Smo protein (Fig. 5D and 6C).

3.4. Effects of resveratrol with or without Shh signaling inhibitor on subsequent development of PA embryos

In experiment 4, I evaluated the effect of 2 μM resveratrol with or without 2 μM cyclopamine treatment during IVM on subsequent embryonic development after PA (Fig. 7A-7D). The results showed a significant increase in cleavage, blastocyst formation rates and total cell numbers in the resveratrol treatment group compared with the control group (87.0%, 30.4% and 55.5 vs. 79.2%, 19.7% and 41.5, respectively). However, the effect was blocked by the combination with cyclopamine (Fig. 7B-7D). Additionally, I tested the expression of genes related to embryo development (*Pou5f1*, *Sox2* and *Nanog*), glucose metabolism (*Glut1*) and apoptosis (*Bax* and *Bcl-2*) in PA-derived blastocysts (Fig. 8A-8C). Blastocysts derived from oocytes matured with resveratrol showed a significant increase in expression of genes related to embryo development (*Pou5f1, Sox2* and *Nanog*), glucose metabolism (*Glut1*) and antiapoptosis (*Bcl-2*), but there was no

effect on expression of *Bax*, a proapoptotic gene. Cyclopamine blocked the resveratrol-induced increase in the expression of all affected genes.

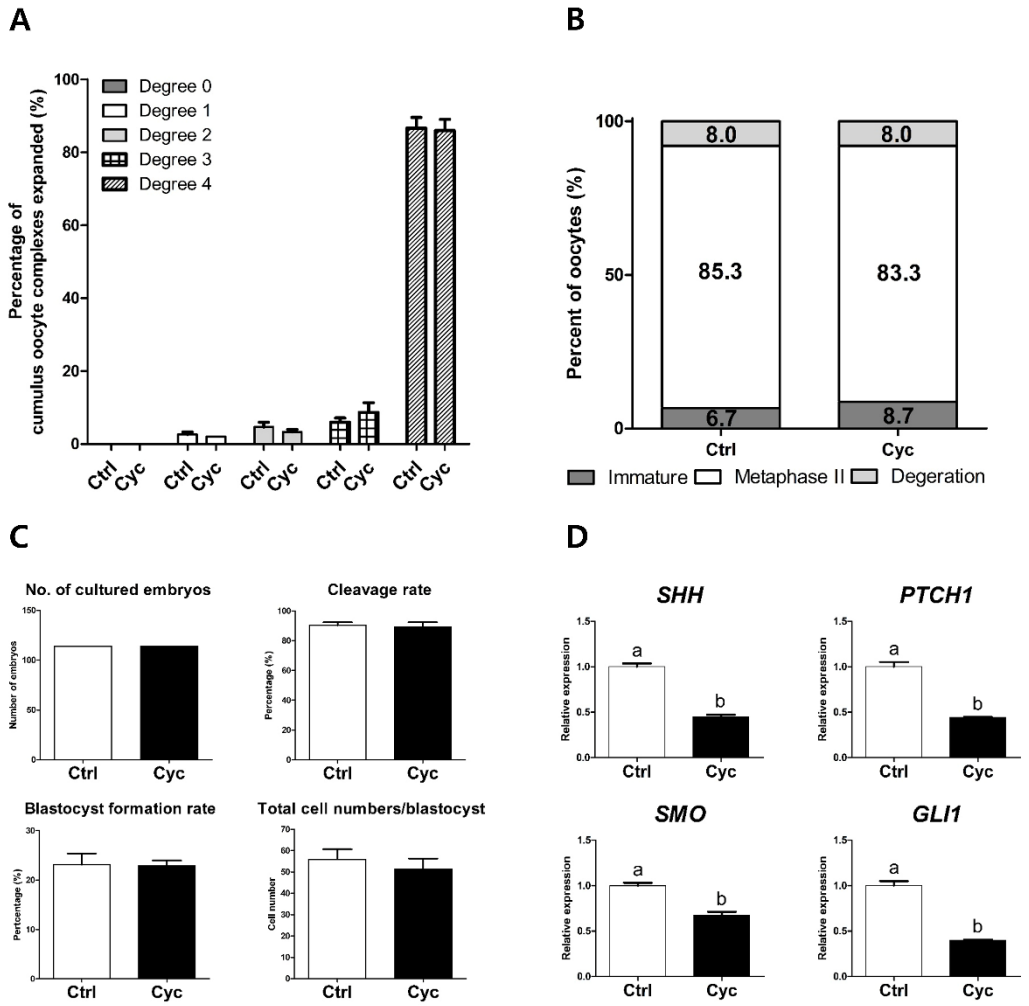


Figure 1. Effect of 2 μM cyclopamine on porcine *in vitro* maturation. (A) Degree of cumulus expansion was classified into five groups, as described previously [169]. (B) Nuclear maturation rate. (C) Developmental competence of porcine oocytes. (D) Relative expression levels of (Shh, Ptch1, Smo and Gli1) in porcine cumulus cells. Data are shown as the means ± SEM. Within each category, groups marked with different letters are significantly different ($P < 0.05$). Ctrl, control; Cyc, 2 μM cyclopamine.

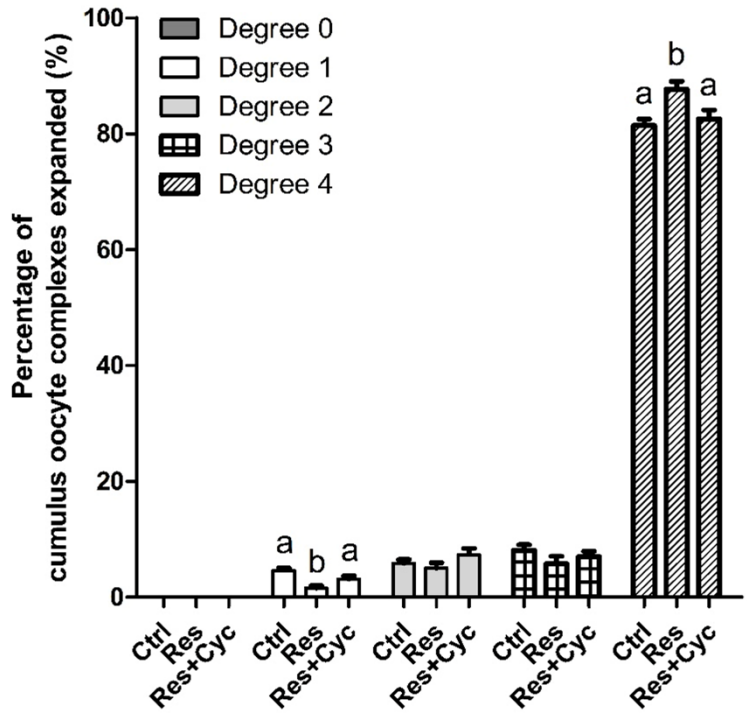


Figure 2. Effect of 2 μM resveratrol with or without 2 μM cyclopamine on cumulus expansion at 44 h of IVM. The degree of cumulus expansion was classified into five groups. Data are shown as the means ± SEM. Within each category, groups marked with different letters are significantly different ($P < 0.05$). A total 774 oocytes were used in five independent replicates. Ctrl, control; Res, 2 μM resveratrol; Cyc, 2 μM cyclopamine.

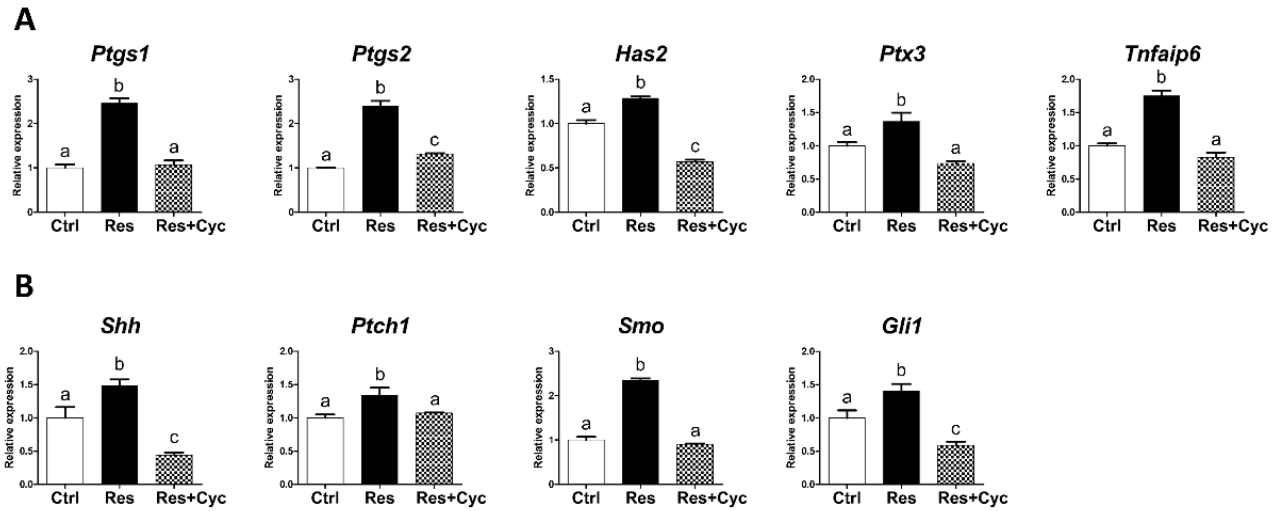


Figure 3. Effect of 2 μM resveratrol with or without 2 μM cyclopamine on gene expression in porcine cumulus cells. (A) Cumulus expansion-related genes (*Ptgs1*, *Ptgs2*, *Has2*, *Ptx3* and *Tnfaip6*). (B) Sonic hedgehog signaling-related genes (*Shh*, *Ptch1*, *Smo* and *Gli1*). Data are shown as the means ± SEM. Within each category, groups marked with different letters are significantly different ($P < 0.05$). The experiment was replicated at least three times. Ctrl, control; Res, 2 μM resveratrol; Cyc, 2 μM cyclopamine

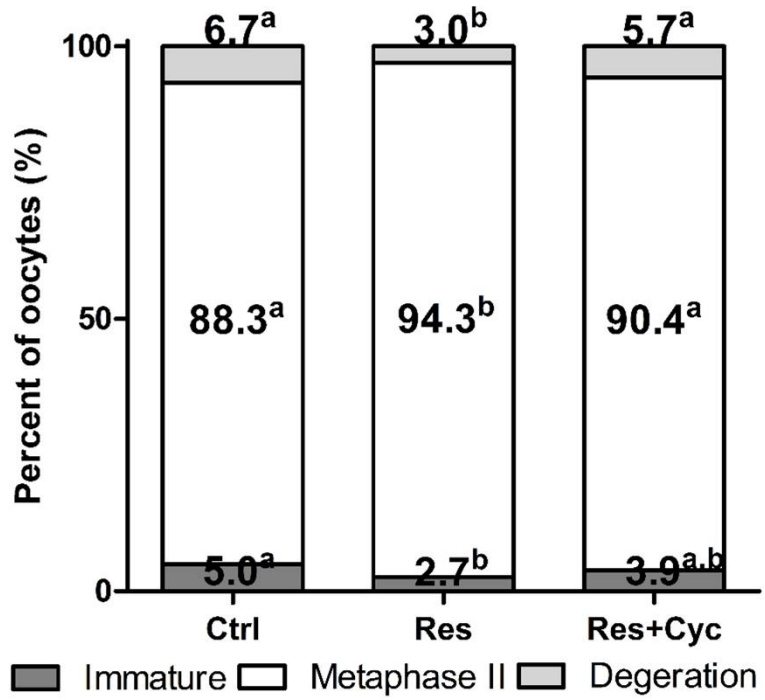


Figure 4. Effect of 2 μM resveratrol with or without 2 μM cyclopamine on the nuclear maturation of porcine oocytes. Data are shown as the means ± SEM. Within each category, groups marked with different letters are significantly different ($P < 0.05$). A total of 783 oocytes were used in five independent replicates. Ctrl, control; Res, 2 μM resveratrol; Cyc, 2 μM cyclopamine

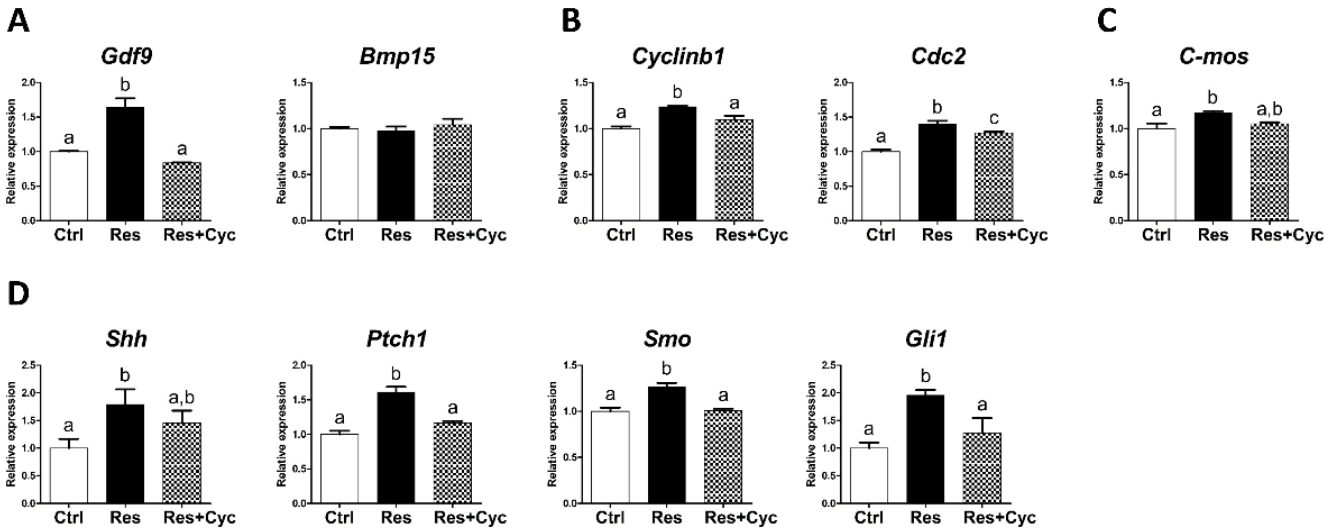


Figure 5. Effect of 2 μM resveratrol with or without 2 μM cyclopamine on gene expression in porcine oocytes. (A) Oocyte competence-related genes (*Gdf9* and *Bmp15*). (B) Maturation promoting factor (MPF)-related genes (*Cyclinb1* and *Cdc2*). (C) Mitogen-activated protein kinases (MAPK) pathway-related gene (*C-mos*). (D) Sonic hedgehog signaling-related genes (*Shh*, *Ptch1*, *Smo* and *Gli1*). Data are shown as the means ± SEM. Within each category, groups marked with different letters are significantly different ($P < 0.05$). The experiment was replicated at least three times. Ctrl, control; Res, 2 μM resveratrol; Cyc, 2 μM cyclopamine

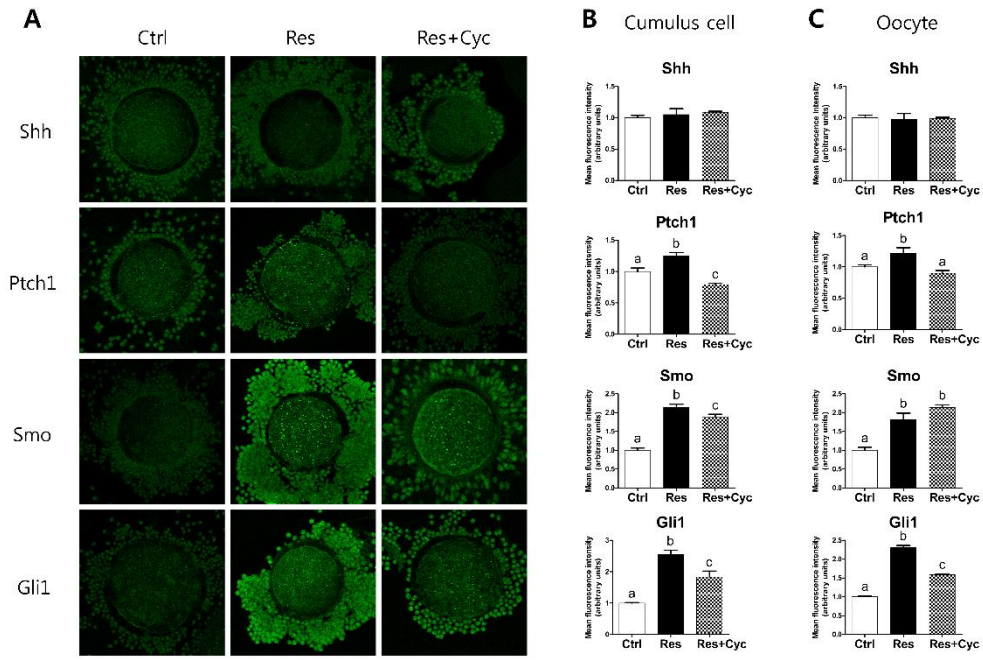


Figure 6. Immunocytochemical analysis of sonic hedgehog signaling proteins in *in vitro* matured porcine cumulus oocyte complexes (COCs). (A) Representative *in vitro* matured porcine COCs of each groups stained with antibodies directed against human Shh, Ptch1, Smo and Gli1. (B and C) Effect of 2 μM resveratrol with or without 2 μM cyclopamine on expression of proteins related to sonic hedgehog signaling in porcine cumulus cells and oocytes, respectively. Data are shown as the means ± SEM. Within each category, groups marked with different letters are significantly different ($P < 0.05$). Ctrl, control; Res, 2 μM resveratrol; Cyc, 2 μM cyclopamine. Original magnification 100×.

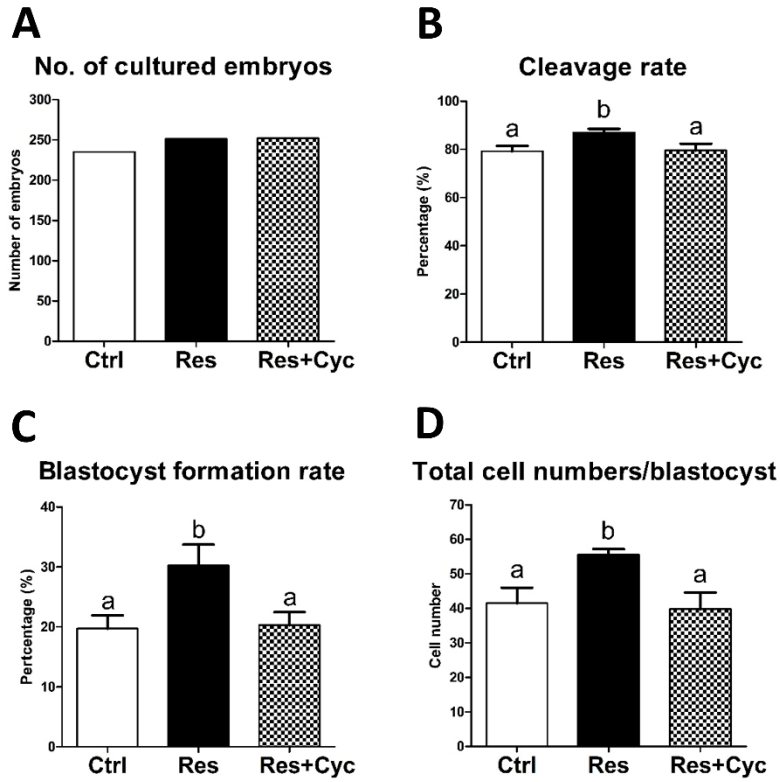


Figure 7. Effect of 2 μM resveratrol with or without 2 μM cyclopamine on subsequent embryo development after parthenogenetic activation. (A) Number of cultured embryos. (B) Cleavage rate. (C) Blastocyst rate. (D) Total cell numbers/blastocyst. Data are shown as the means ± SEM. Within each category, groups marked with different letters are significantly different ($P < 0.05$). A total of 738 oocytes were used in five independent replicates. Ctrl, control; Res, 2 μM resveratrol; Cyc, 2 μM cyclopamine.

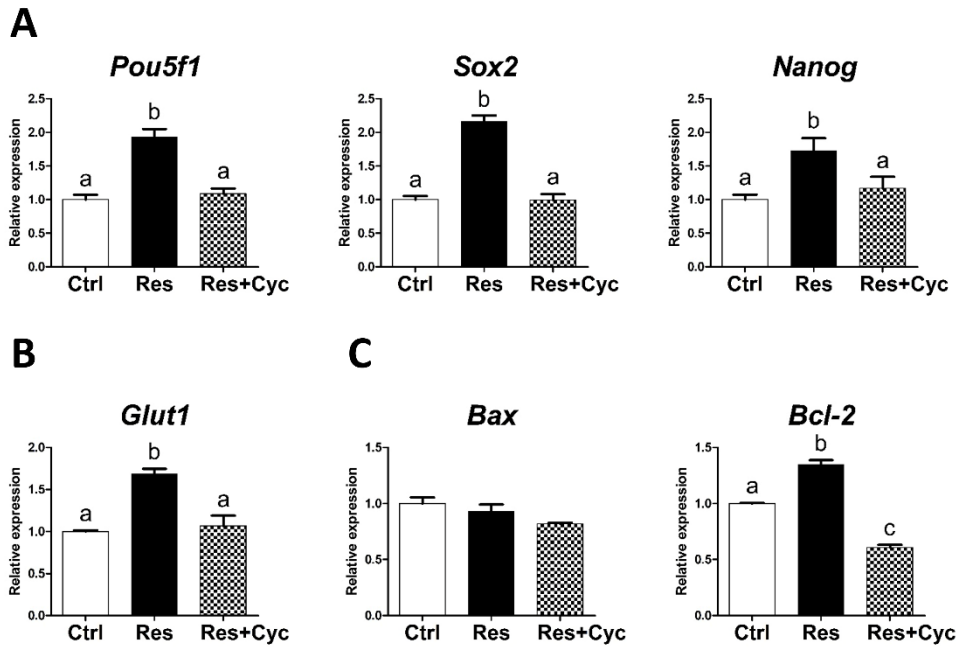


Figure 8. Effect of 2 μM resveratrol with or without 2 μM cyclopamine on gene expression in blastocysts derived from parthenogenetically activated oocytes. (A) Developmental competence-related genes (*Pou5f1*, *Sox2* and *Nanog*). (B) Glucose metabolism-related gene (*Glut1*). (C) Apoptosis-related genes (*Bax* and *Bcl-2*). Data are shown as the means ± SEM. Within each category, groups marked with different letters are significantly different ($P < 0.05$). The experiment was replicated at least three times. Ctrl, control; Res, 2 μM resveratrol; Cyc, 2 μM cyclopamine.

4. Discussion

Resveratrol has been used to improve the maturation of oocytes *in vitro* in various species and many studies have attributed its beneficial actions on IVM to indirect antioxidant properties of resveratrol [91, 104, 106, 107]. However, direct actions of resveratrol on COCs have not been well elucidated. Here, I demonstrated that resveratrol stimulated porcine cumulus expansion, nuclear maturation of oocytes and their subsequent embryo development. Furthermore, I also found direct evidence that these beneficial effects of resveratrol may be attributed to activation of the Shh signaling pathway.

To elucidate underlying mechanism by which resveratrol directly acts on porcine COCs, it is required to understand the biological activities of resveratrol and the *in vivo* physiology of oocyte maturation. Recently, several lines of evidence suggested the involvement of Shh signaling in biological activities of resveratrol. Resveratrol protects against ischemic injury after stroke through Shh signaling [164] and it can induce differentiation of bone mesenchymal stem cells into neuronal-like cells by activating a Shh signaling pathway in primary cilia [171]. In addition, several studies verified that polydatin, a glucoside of resveratrol, definitively impacted the Shh signaling pathway to exert various biological activities in different models [172-174].

Considering the *in vivo* physiology of oocyte maturation, Shh is important signaling during ovarian follicular development [132]. The regulation of meiotic arrest and oocyte maturation are mediated by follicular somatic cell compartments such as the granulosa

and cumulus cells surrounding the oocytes [175]. Expansion of the granulosa and cumulus cell layers, which is supposed to be closely related to Hh signaling [132], plays an important role in the maturation of oocytes [176]. Furthermore, recently, targets (Ptch, Smo and Gli1) of Shh signaling were found in the porcine granulosa and cumulus cell layers and the beneficial actions of the activated Shh signaling pathway on porcine IVM were demonstrated [139]. In this regard, I hypothesized that resveratrol-induced improvements in oocyte maturation and subsequent embryo development might be closely related to a Shh signaling pathway.

In the present study, supplementation of resveratrol to the IVM medium significantly increased cumulus expansion of porcine COCs and expression of genes related to cumulus expansion (*Ptgs1*, *Ptgs2*, *Has2*, *Ptx3* and *Tnfaip6*) in cumulus cells. As optimum expansion of the cumulus layer is necessary for proper maturation of oocytes [177], expansion itself has been used as a gross indicator of oocyte maturation [178] and higher rates of subsequent embryo development have been attributed to cumulus expansion [179]. To determine the involvement of the Shh signaling pathway in these beneficial effects of resveratrol on cumulus expansion, cyclopamine, an inhibitor of Shh signaling through direct interaction with Smo, was applied to block the Shh signaling pathway. Cyclopamine has been widely used to elucidate the relationship between drugs or chemicals and Shh signaling [174, 180, 181]. As 2 μM cyclopamine did not affect cumulus expansion, oocyte nuclear maturation and subsequent embryo development, I could exclude the possibility that reduction in resveratrol-induced improvements can occur through the inhibition of endogenous Shh signaling by cyclopamine. Concomitant

supplementation of cyclopamine to resveratrol significantly blocked the beneficial effects of resveratrol in terms of the degree of cumulus expansion and expression of all investigated genes related to cumulus expansion (*Ptgs1*, *Ptgs2*, *Has2*, *Ptx3* and *Tnfaip6*). These results suggested that the ability of resveratrol to promote cumulus expansion is due to activation of Shh signaling. To clarify the relationship between the beneficial effects of resveratrol on cumulus expansion and Shh signaling, expression of Shh signaling genes (*Shh*, *Ptch1*, *Smo* and *Gli1*) and proteins (Shh, Ptch1, Smo and Gli1) in cumulus cells was also investigated. Resveratrol significantly increased the expression of Shh signaling genes (*Shh*, *Ptch1*, *Smo* and *Gli1*) and proteins (Ptch1, Smo and Gli1), which was inhibited in the presence of cyclopamine. Based on these results, I concluded that the Shh signaling pathway mediated the effect of resveratrol on porcine cumulus expansion *in vitro*.

Regarding a direct action of resveratrol on porcine oocytes, resveratrol significantly improved oocyte nuclear maturation. Furthermore, it significantly increased the expression of genes related to oocyte competence (*Gdf9*), MPF (*Cyclinb1* and *Cdc2*) and the MAPK pathway (*C-mos*) in oocytes. Facilitation of the meiotic progression of oocytes is regulated by MPF and MAPK-mediated phosphorylation and dephosphorylation [182]. Therefore, these gene expression analysis results supported the increase in nuclear maturation by resveratrol. However, cyclopamine blocked these beneficial effects of resveratrol on oocytes. The concomitant addition of cyclopamine to resveratrol prevented improvements in nuclear maturation and expression of genes (*Gdf9*, *Cyclinb1* and *Cdc2*). Moreover, cyclopamine blocked the resveratrol-induced increase

in expression of Shh signaling genes (*Shh*, *Ptch1*, *Smo* and *Gli1*) and proteins (*Ptch1*, *Smo* and *Gli1*), except for expression of a Shh gene and a Smo protein. Based on these results, I conclude that resveratrol influenced porcine oocyte maturation at least partly through the Shh signaling pathway.

Resveratrol supplementation during IVM improved subsequent embryo development in terms of cleavage, blastocyst formation rates and total cell numbers. Furthermore, it significantly increased expression of genes related to developmental competence (*Pou5f1*, *Sox2* and *Nanog*), glucose metabolism (*Glut1*) and antiapoptosis (*Bcl-2*). Because oocyte quality is an important determinant of the final outcome of *in vitro* embryo production [183], improvement in the IVM environment by resveratrol supplementation is thought to promote subsequent embryonic development. In summary, resveratrol improved subsequent embryo development in terms of cleavage, blastocyst formation rates, total cell numbers, and expression of developmentally important genes in the blastocysts, however, simultaneous addition of cyclopamine to resveratrol blocked these improvements. These results indicate that the ability of resveratrol to promote porcine IVM and subsequent embryo development could be through activation of Shh signaling.

In conclusion, this study provides the first evidence that the Shh signaling pathway mediates the beneficial actions of resveratrol on cumulus expansion, oocyte nuclear maturation and subsequent early embryo development. These findings will be helpful in explaining the underlying mechanism by which resveratrol acts directly on porcine COCs and in opening new avenues for potential clinic applications of resveratrol.

However, further studies are needed to elucidate the complete mechanism by which resveratrol activates Shh signaling in porcine COCs.

Chapter II. Melatonin influences the sonic hedgehog signaling pathway in porcine cumulus oocyte complexes

1. Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine), a natural hormone produced in the mammalian pineal gland [184] and peripheral reproductive system [185] such as the cells of the ovary including granulosa cells, cumulus cells and oocytes, exerts various biological activities, including antioxidation [186], immune defense [187] and anti-cancer effects [188]. This hormone also affects reproduction; for example, it is generally accepted that melatonin influences ovarian function directly [113]. Expression of melatonin receptor in cells of the ovary [114, 115] and also the presence of melatonin in ovarian follicular fluid that even exceeds blood levels [116, 185] support a local role of melatonin in reproductive processes. Furthermore, many studies have reported beneficial effects of melatonin on IVM of oocytes in mice [117], humans [118], cattle [119-122] and pigs [92, 123-125].

Multiple actions of melatonin on various physiological processes are mediated by melatonin membrane receptors [189, 190] or by an indirect antioxidant mechanism scavenging free radicals [191, 192]. Previously, beneficial effects of melatonin on IVM of oocytes have been attributed mainly to the indirect antioxidant effects of melatonin [92, 117, 122, 193]. However, little information is available on the direct action of melatonin on IVM. To elucidate this, a better understanding of the *in vivo* physiology of oocyte maturation is required.

During follicular development, communication among the oocyte, granulosa cell and theca cell compartments regulates each other's proliferation and differentiation [165] via signaling pathways which are necessary for development and patterning in many tissues [132]. The Hh signaling pathway was first identified in Drosophila melanogaster [127] and is the well-known morphogen controlling gonad development and basic embryonic developmental processes [129]. In vertebrates, there are three Hh members- Shh, Ihh and Dhh [130-132]. When these ligands bind to the Ptch receptor on the cell surface, inhibition of Smo, the seven-transmembrane G-protein-coupled coreceptor, is released. Then, activation of Smo subsequently activates the Gli1 transcription factor, which regulates cell patterning, proliferation, migration and differentiation during development [163]. Recently, targets (Ptch, Smo and Gli1) of Hh signaling were found to exist in the granulosa and cumulus cell layer of porcine oocytes and addition of Shh protein during IVM promoted oocyte maturation and subsequent *in vitro* development [139]. These reports demonstrated that Shh signaling is important for follicular development and oocyte maturation.

Considering the *in vivo* physiology of oocyte maturation, melatonin supplementation during IVM is expected to influence the activation of fundamental signaling pathways such as Shh. The aim of this study, therefore, was to determine the relationship between beneficial effects of melatonin on porcine IVM and Shh signaling pathway. In the present study, Shh signaling was inhibited using cyclopamine, an inhibitor of Smo (one of the Shh signaling molecules) [168], to confirm whether or not Shh signaling is induced by melatonin. I evaluated the effects of melatonin with or without cyclopamine during IVM

on cumulus expansion, oocyte nuclear maturation, subsequent embryonic development and the expression of genes and proteins related to Shh signaling.

2. Materials and methods

2.1. Oocyte recovery and IVM

Procedures for IVM were described in general methodology.

2.2 Cumulus expansion assessment

The proportions of oocytes exhibiting cumulus expansion were determined at the endpoint of IVM. The degree of cumulus expansion was assessed by the morphology of the COCs as described previously [169]. Briefly, a degree of 0 indicated no expansion, characterized by detachment of cumulus cells from the oocyte to assume a flattened monolayer of fibroblastic appearance, leaving a partially or fully denuded oocyte. A degree of 1 indicated no expansion but cumulus cells are spherical, and remain compacted around the oocyte. For degree 2 complexes, only the outermost layers of cumulus cells have expanded, while degree 3 complexes have all cell layers except the corona radiata (cells most proximal to the oocyte) prominently expanded, and a degree of 4 indicated the maximum degree of expansion including the corona radiata.

2.3. Assessment of nuclear maturation

After 42-44 h of IVM, oocytes were sampled to analyze nuclear maturation. Samples of oocytes were denuded by gently pipetting with 0.1% hyaluronidase in TALP medium and washed three times in TALP medium. The denuded oocytes were evaluated with a microscope (TE2000-S, Nikon Corp.) and classified as immature (without polar body extrusion), degenerate, or at metaphase II (with first polar body extrusion).

2.4. Parthenogenetic activation of oocytes

After 42-44 h of IVM culture, COCs were denuded by gently pipetting with 0.1% hyaluronidase, washed three times in TALP medium, and then gradually equilibrated in activation medium consisting of 0.28 M mannitol, 0.5 mM HEPES, 0.1 mM $CaCl_2$ and 0.1 mM $MgSO_4$. For activation, denuded oocytes with homogeneous cytoplasm were placed between electrodes covered with activation medium in a chamber connected to a BTX Electro-Cell Manipulator 2001 (BTX Inc.). Oocytes were activated with a single DC pulse of 1.5 kV/cm for 60 μs. Then, electrically-activated oocytes were washed three times in fresh PZM-5 (Funakoshi Corporation), placed into 500 μL PZM-5 wells and cultured at 39 °C in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2 for 7 days.

2.5. Embryo evaluation and total cell count after PA

The day of PA was considered Day 0. Embryos were evaluated under a stereomicroscope for cleavage on Day 2 (48 h). Evenly-cleaved embryos were classified into three groups (2 to 3, 4 to 5, and 6 to 8 cells). Blastocyst formation was assessed on Day 7 (168 h) after PA. To count the total cell numbers of blastocysts, they were collected on Day 7, washed in TALP medium, and stained with 5 μg/mL Hoechst-33342 for 10 min. After a final wash in TALP medium, the blastocysts were mounted on glass slides in a drop of 100% glycerol, compressed gently with a cover slip, and observed under a fluorescence microscope (Nikon Corp.) at magnification × 400 to count cell nuclei.

2.6. Gene expression analysis by real-time PCR

Each real-time PCR was performed with isolated cumulus cells, mature oocytes, and PA-derived blastocysts. Three groups (control, melatonin and melatonin with cyclopamine) of cumulus cells, oocytes and PA-derived blastocysts were separately sampled under a stereomicroscope to study gene expression. All samples were stored at -80 °C until analysis. Total RNA was extracted using the easy-spin Total RNA Extraction Kit (iNtRON), according to the manufacturer's protocol, and the total RNA concentration was quantified using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). The cDNA was produced using amfiRivert cDNA Synthesis Platinum Master Mix (GenDEPOT) according to the manufacturer's protocol. A PCR plate (MicroAmp optical 96-well reaction plate, Applied Biosystems) was made by adding 1 μL cDNA, 0.4 μL (10 pmol/μL) forward primer, 0.4 μL (10 pmol/μL) reverse primer, 10 μL SYBR Premix Ex Taq (Takara), and 8.2 μL of Nuclease-free water (Ambion). The reactions were carried out for 40 cycles and the cycling parameters were as follows: denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min. All oligonucleotide primer sequences are presented in Table 3. The expression of each target gene was quantified relative to that of the internal control gene (*GAPDH*) using the equation, $R = 2^{-[\Delta Ct\ sample\ -\ \Delta Ct\ control]}$.

2.7. Immunocytochemical staining

In vitro matured COCs were washed three times in PBS containing 0.2% PVA and fixed with 4% paraformaldehyde (w/v) in PBS for 1 h at room temperature. After washing three times in PBS with 0.2% PVA, COCs were permeated with 1% (v/v) Triton X-100 in PBS for 30 min. Then COCs were washed three times in PBS with 0.2% PVA and blocked with 2% BSA in PBS overnight at 4 °C. The COCs were incubated with primary antibodies for Shh (1:200; sc-365112; Santa Cruz Biotechnology, Inc.), Ptch1 (1:200; sc-9016; Santa Cruz), Smo (1:200; sc-13943; Santa Cruz), or Gli1 (1:200; sc-20687; Santa Cruz) at 37 °C for 3 h. After that, they were washed three times in PBS with 2% BSA and then incubated with a secondary fluorescein isothiocyanate-conjugated anti-mouse monoclonal antibody (1:200; Jackson ImmunoResearch Laboratories Inc.) or anti-rabbit polyclonal antibody (1:200; ab6717, Abcam) for 1 h (in darkness). After washing three times in PBS with 2% BSA, samples were mounted on glass slides. Images were captured with a confocal laser-scanning microscope (SP8 X, Leica). The intensities of Shh, Ptch1, Smo and Gli1 were measured by Image J software (version 1.46r; National Institutes of Health).

2.8. Experimental design

There were three experimental groups: (1) control, (2) melatonin, and (3) melatonin with cyclopamine (Shh signaling inhibitor). The agents were added to the IVM medium during the entire maturation period of 44 h. The concentrations of melatonin (10^{-9} M) [123, 125] and cyclopamine (2 μM) [139] were set according to previous studies. In experiment 1, I evaluated the degree of cumulus expansion, expression of genes related

to cumulus expansion and Shh signaling and expression of proteins related to Shh signaling in cumulus cells. In experiment 2, I investigated the oocyte maturation rate, expression of genes related to oocyte competence, MPF, MAPK pathway and Shh signaling, and expression of proteins related to Shh signaling in oocytes. In experiment 3, I assessed *in vitro* development of PA embryos and expression of genes related to developmental competence, glucose metabolism and apoptosis in blastocysts derived from PA oocytes. Experiment 4 was designed to determine the presence of transcripts of melatonin receptor 1 (*MT1*) and 2 (*MT2*) on oocytes and cumulus cells and to compare expression levels of *MT1* and *MT2* transcripts in cumulus cells.

2.9. Statistical analysis

Procedures for statistical analysis were described in general methodology.

Table 3. List of real-time PCR primers

Gene	Primer sequences (5'-3')		Product size (bp)	GenBank accession number
	Forward	Reverse		
GAPDH	GTCGGTTGTGGATCTGACCT	TTGACGAAGTGGTCGTTGAG	207	NM_001206359
Shh	ACTGACCCCTTTAGCCTACA	GTCGGCTCCAGTGTTTTCTT	172	NM_001244513
Ptch1	CTCGGGAAACGAGAGAATAC	AGTAGTGCAGCCACATTTTG	198	XM_003482800
Smo	AGAGATACGTGCTCGCTCTT	CTGTCTGGTGCTTGAAACTG	201	XM_003134680
Gli1	AGAGGGACAGCTCTGAACAC	GCTACGTCTCTTCCTCCTGA	199	NM_001256593
Ptgs1	CAACACGGCACACGACTACA	CTGCTTCTTCCCTTTGGTCC	121	XM_001926129
Ptgs2	ACAGGGCCATGGGGTGGACT	CCACGGCAAAGCGGAGGTGT	194	NM_214321
Has2	AGTTTATGGGCAGCCAATGTAGTT	GCACTTGGACCGAGCTGTGT	101	AB050389
Ptx3	GGCCAGGGATGAATTTTAC	GCTATCCTCTCCAACAAGTGA	185	NM_001244783
Tnfapi6	AGAAGCGAAAGATGGGATGCT	CATTTGGGAAGCCTGGAGATT	106	NM_001159607
Gdf9	CAGTCAGCTGAAGTGGGACA	TGGATGATGTTCTGCACCAT	135	AY626786
Bmp15	CCTCCATCCTTTCCAAGTCA	GTGTAGTACCCGAGGGCAGA	112	NM_001005155
Cyclinb1	CAACTGGTTGGTGTCACTGC	TTCCATCTGCCTGATTTGGT	126	L48205
Cdc2	GGGCACTCCCAATAATGAAGT	GTTCTTGATACAACGTGTGGGAA	260	AB045783
C-mos	GGGAGCAACTGAACTTGGAG	AGAATGTTCGCTGGCTTCAG	115	NM_001113219
Pou5f1	TTTGGGAAGGTGTTCAGCCAAACG	TCGGTTCTCGATACTTGTCCGCTT	198	NM_001113060

Sox2	ATGCACAACTCGGAGATCAG	TATAATCCGGGTGCTCCTTC	130	NM_001123197
Nanog	GGTTTATGGGCCTGAAGAAA	GATCCATGGAGGAAGGAAGA	98	NM_001129971
Glut1	GCTTCCAGTATGTGGAGCAA	AAGCAATCTCATCGAAGGTC	132	XM_013977359
Bax	TGCCTCAGGATGCATCTACC	AAGTAGAAAAGCGCGACCAC	199	XM_003127290
Bcl2	AGGGCATTCAGTGACCTGAC	CGATCCGACTCACCAATACC	193	NM_214285
MT1	ATACGACAGGTGGTACAGCA	ACTGACGGACTGTGCAAACGT	150	XM_013985006
MT2	CGGTCGTGTGCTTCTGTTACC	AGCAGACGGCGAAGATCA	151	XM_013979266

3. Results

3.1. Effects of melatonin with or without Shh signaling inhibitor on cumulus expansion

I evaluated the effects of 10^{-9} M melatonin with or without 2 μM cyclopamine treatment during IVM on cumulus expansion, expression of genes related to cumulus expansion (*Ptgs1*, *Ptgs2*, *Has2*, *Ptx3* and *Tnfaip6*) and expression of Shh signaling genes (*Shh*, *Ptch1*, *Smo* and *Gli1*) and proteins (Shh, Ptch1, Smo and Gli1). As shown in Figure 9, melatonin significantly increased the proportion of COCs exhibiting complete cumulus expansion (degree 4) and showed a significant decrease in the proportion of degree 3 compared to the control. However, these effects were inhibited by addition of cyclopamine. In addition, increased expression of genes related to cumulus expansion (*Ptgs1*, *Ptgs2* and *Has2*) after treatment with melatonin was also blocked by cyclopamine treatment (Figure 10A). Although expression of *Ptx3* and *Tnfaip6* was also significantly increased by melatonin, their increase was not blocked by cyclopamine. To establish whether melatonin-mediated cumulus expansion was through Shh signaling, expression of Shh signaling genes and proteins was examined. Melatonin significantly increased expression of Shh signaling genes (*Shh*, *Ptch1*, *Smo* and *Gli1*) and proteins (Ptch1, Smo and Gli1) in cumulus cells. However, cyclopamine inhibited the melatonin-induced increase in expression of Shh signaling genes and proteins (Figure 10B and 13B). Expression patterns of Shh signaling genes and proteins, which were increased by melatonin and blocked by addition of cyclopamine, were similar to those observed from

the proportion of COCs exhibiting complete cumulus expansion (degree 4) and expression of cumulus expansion genes (*Ptgs1*, *Ptgs2* and *Has2*).

3.2. Effects of melatonin with or without Shh signaling inhibitor on oocyte nuclear maturation

I evaluated the effect of 10^{-9} M melatonin with or without 2 μM cyclopamine treatment during IVM on oocyte nuclear maturation. The rates of oocyte maturation ranged from 81.9% to 90.6% and no significant difference was observed among the groups (Figure 11). Additionally, I examined the expression of genes related to oocyte competence (*Gdf9* and *Bmp15*), MPF (*Cyclinb1* and *Cdc2*), the MAPK pathway (*C-mos*) and expression of Shh signaling genes and proteins in oocytes (Figure 12 and 13C). Expression of an oocyte competence gene (*Gdf9*) and a MAPK pathway gene (*C-mos*) was significantly increased by melatonin treatment, but this increase was blocked in the presence of cyclopamine (Figure 12A and 12C). However, no significant difference in expression of MPF genes (*Cyclinb1* and *Cdc2*) was observed between the control and melatonin treatment groups (Figure 12B). Even in expression of Shh signaling genes, only *Gli1* expression was significantly increased by melatonin treatment, and again cyclopamine depressed this increase (Figure 12D). As for protein expression, melatonin significantly increased expression of Ptch1, Smo and Gli1 in oocytes (Figure 13C). However, only Gli1 expression was blocked by cyclopamine treatment.

3.3. Effects of melatonin with or without Shh signaling inhibitor on subsequent embryo development

The effect of 10^{-9} M melatonin with or without 2 μM cyclopamine on subsequent embryonic development after PA was investigated (Figure 14). The results revealed a significant increase in blastocyst formation and total cell numbers in the melatonin treatment group compared with the control group (32.0% and 56.7 vs. 21.9% and 41.0, respectively), whereas no differences in blastocyst formation rates and total cell numbers were observed between the melatonin with cyclopamine and control groups (21.4% and 40.0 vs. 21.9% and 41.0, respectively). Furthermore, I examined the expression of genes related to embryo development (*Pou5f1*, *Sox2*, and *Nanog*), glucose metabolism (*Glut1*) and apoptosis (*Bax* and *Bcl-2*) in PA-derived blastocysts (Figure 15). Blastocysts derived from the melatonin treatment group showed a significant increase in expression of embryo development genes (*Sox2* and *Nanog*) and a decrease in proapoptotic gene (*Bax*) expression, but there was no effect on *Pou5f1*, *Glut1* and *Bcl-2* mRNA expression. Cyclopamine blocked the increased expression of *Sox2* from melatonin treatment. However, expression of *Nanog* and *Bax* was not affected by cyclopamine.

3.4. Effects of melatonin with or without Shh signaling inhibitor on subsequent embryo development

To investigate the reason for more beneficial effects of melatonin on cumulus cells than on oocytes, I investigated the presence of transcripts of melatonin receptors (*MT1* and *MT2*) in porcine cumulus cells and oocytes (Figure 16A). The expected size of PCR

products for *MT1* was 150 bp and for *MT2*, it was 151 bp. Both *MT1* and *MT2* transcripts were detected in porcine cumulus cells. However, *MT1* transcript expression was not detected in porcine oocytes, which is consistent with a previous study [92]. Additionally, I compared the expression of *MT1* and *MT2* in cumulus cells using real-time PCR. As shown in Figure 16B and 16C, melatonin significantly increased *MT1* and *MT2* mRNA transcript levels in cumulus cells compared with the control group, regardless of the addition of cyclopamine.

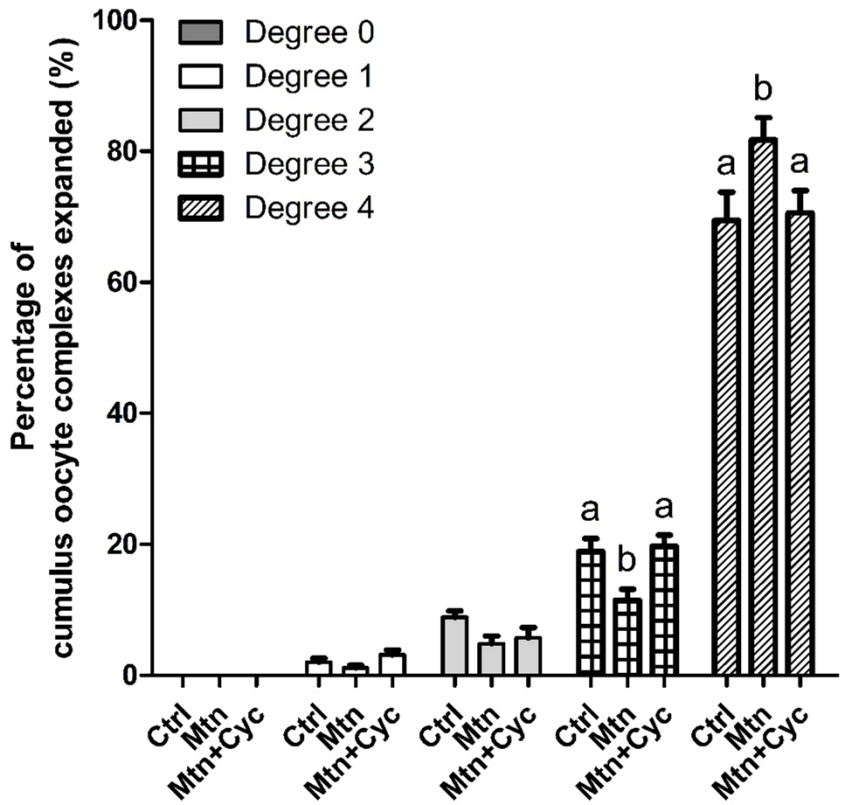


Figure 9. Effect of 10^{-9} M melatonin with or without 2 μM cyclopamine on cumulus expansion at 44 h of IVM. The degree of cumulus expansion was classified into five groups. Data are shown as the means ± SEM. Within each category, groups marked with different letters are significantly different ($P < 0.05$). A total of 1041 oocytes were used in seven independent replicates. Ctrl, control; Mtn, 10^{-9} M melatonin; Cyc, 2 μM cyclopamine.

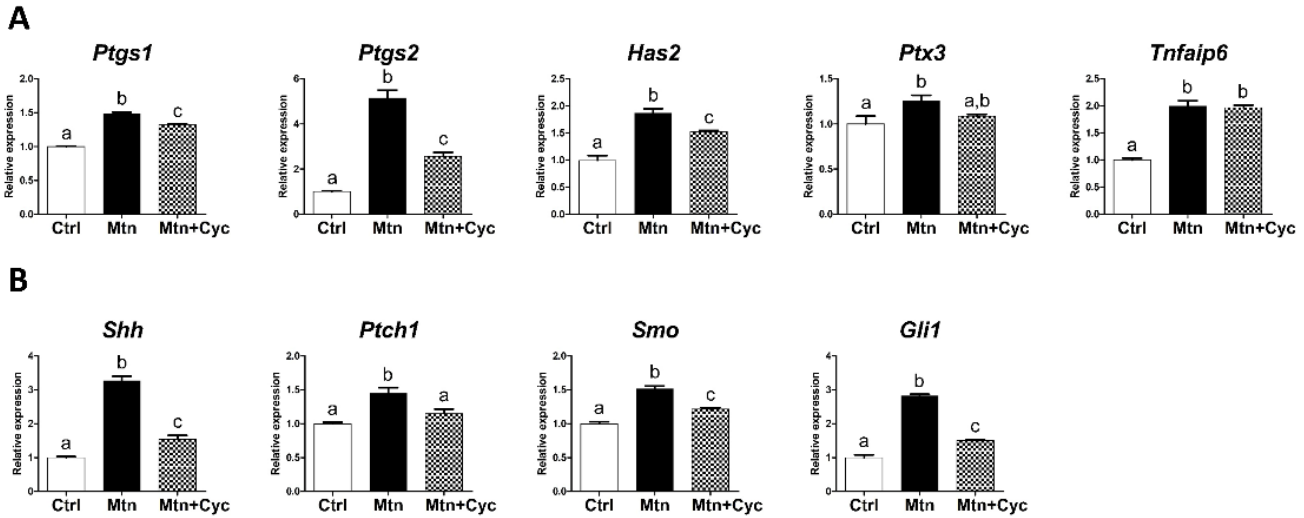


Figure 10. Effect of 10^{-9} M melatonin with or without 2 μM cyclopamine on gene expression in porcine cumulus cells. (A) Cumulus expansion-related genes (*Ptgs1*, *Ptgs2*, *Has2*, *Ptx3* and *Tnfaip6*). (B) Sonic hedgehog signaling-related genes (*Shh*, *Ptch1*, *Smo* and *Gli1*). Data are shown as the means ± SEM. Within each category, groups marked with different letters are significantly different ($P < 0.05$). The experiment was replicated at least three times. Ctrl, control; Mtn, 10^{-9} M melatonin; Cyc, 2 μM cyclopamine

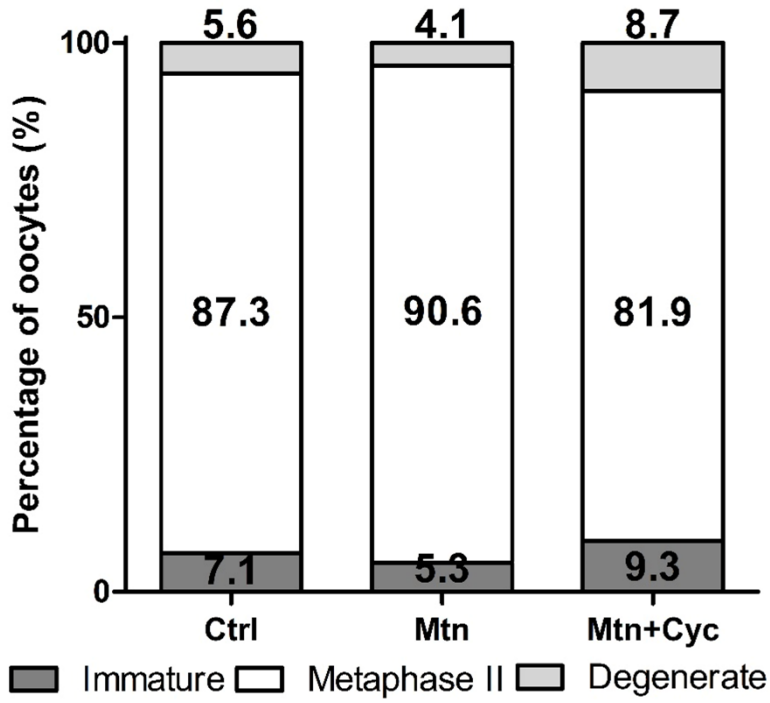


Figure 11. Effect of 10^{-9} M melatonin with or without 2 μM cyclopamine on the nuclear maturation of porcine oocytes. A total of 798 oocytes were used in five independent replicates. Ctrl, control; Mtn, 10^{-9} M melatonin; Cyc, 2 μM cyclopamine.

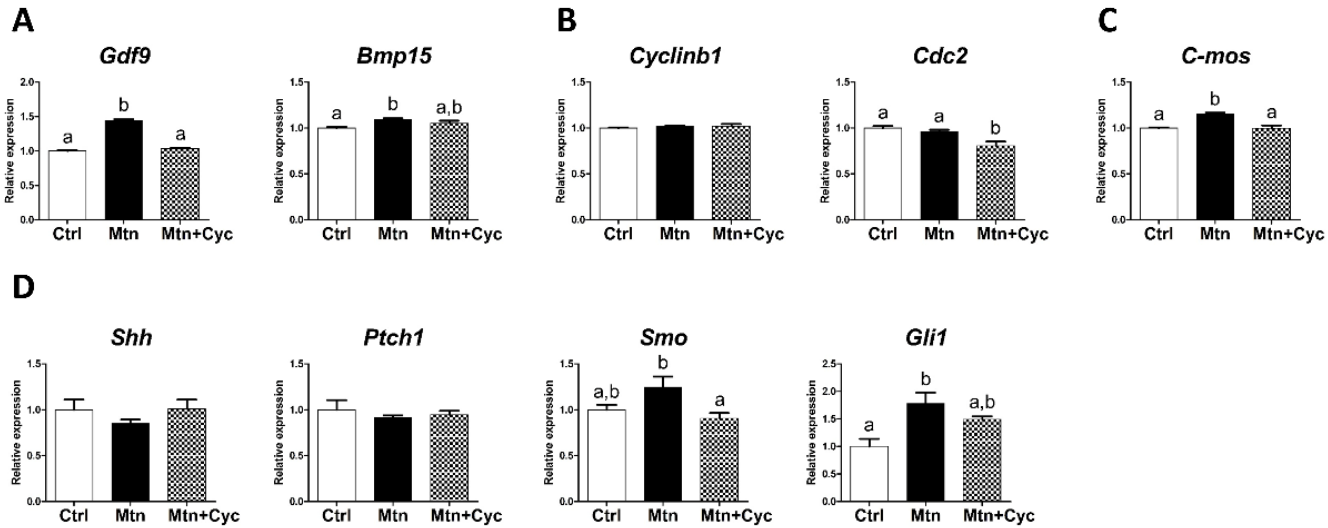


Figure 12. Effect of 10^{-9} M melatonin with or without 2 μM cyclopamine on gene expression in porcine oocytes. (A) Oocyte competence-related genes (*Gdf9* and *Bmp15*). (B) Maturation promoting factor (MPF)-related genes (*Cyclinb1* and *Cdc2*). (C) Mitogen-activated protein kinases (MAPK) pathway-related gene (*C-mos*). (D) Sonic hedgehog signaling-related genes (*Shh*, *Ptch1*, *Smo* and *Gli1*). Data are shown as the means ± SEM. Within each category, groups marked with different letters are significantly different ($P < 0.05$). The experiment was replicated at least three times. Ctrl, control; Mtn, 10^{-9} M melatonin; Cyc, 2 μM cyclopamine.

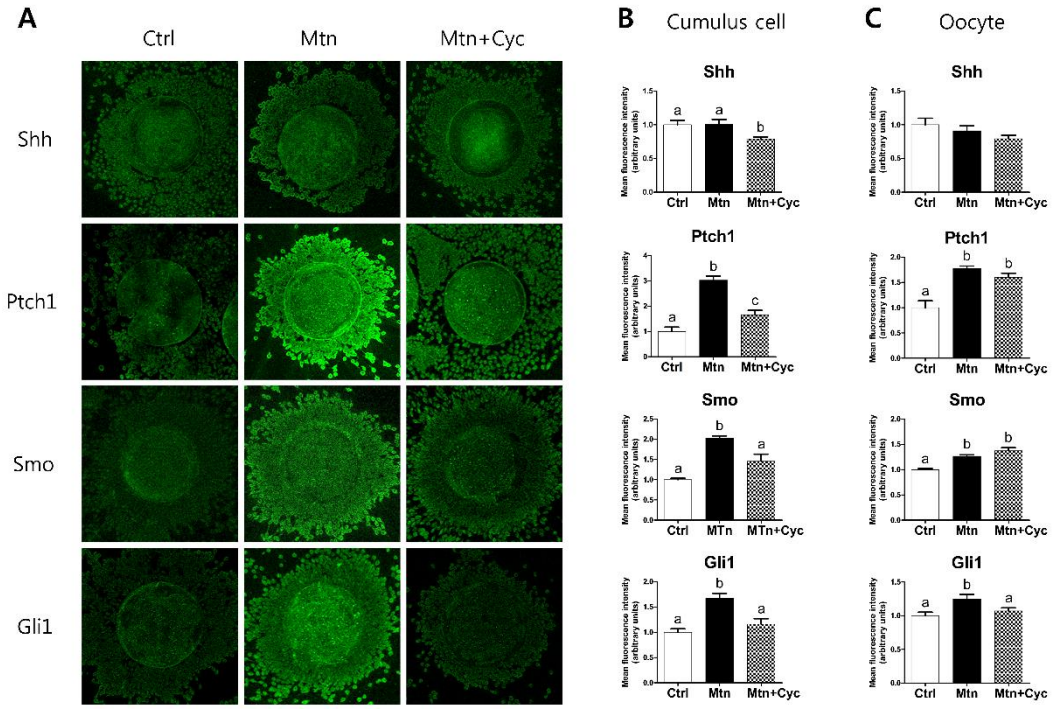


Figure 13. Immunocytochemical analysis of sonic hedgehog signaling proteins in *in vitro* matured porcine cumulus oocyte complexes (COCs). (A) Representative *in vitro* matured porcine COCs of each group stained with antibodies directed against human Shh, Ptch1, Smo and Gli1. (B and C) Effect of 10^{-9} M melatonin with or without 2 μM cyclopamine on expression of proteins related to sonic hedgehog signaling in porcine cumulus cells and oocytes, respectively. Data are shown as the means ± SEM. Within each category, groups marked with different letters are significantly different ($P < 0.05$). Ctrl, control; Mtn, 10^{-9} M melatonin; Cyc, 2 μM cyclopamine. Original magnification 100×.

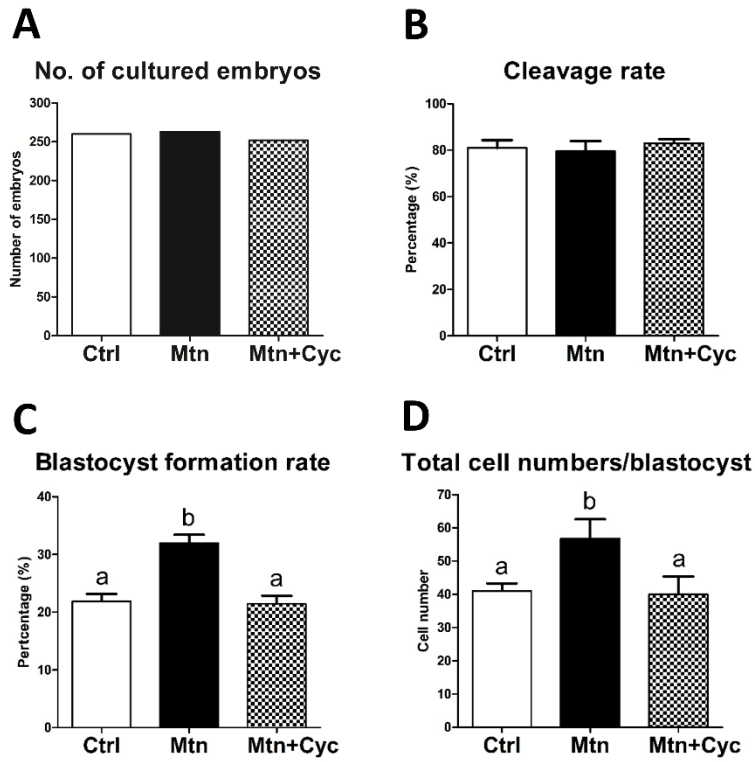


Figure 14. Effect of 10^{-9} M melatonin with or without 2 μM cyclopamine on subsequent embryo development after parthenogenetic activation. (A) Number of cultured embryos. (B) Cleavage rate. (C) Blastocyst rate. (D) Total cell numbers/blastocyst. Data are shown as the means ± SEM. Within each category, groups marked with different letters are significantly different ($P < 0.05$). A total of 775 oocytes were used in seven independent replicates. Ctrl, control; Mtn, 10^{-9} M melatonin; Cyc, 2 μM cyclopamine.

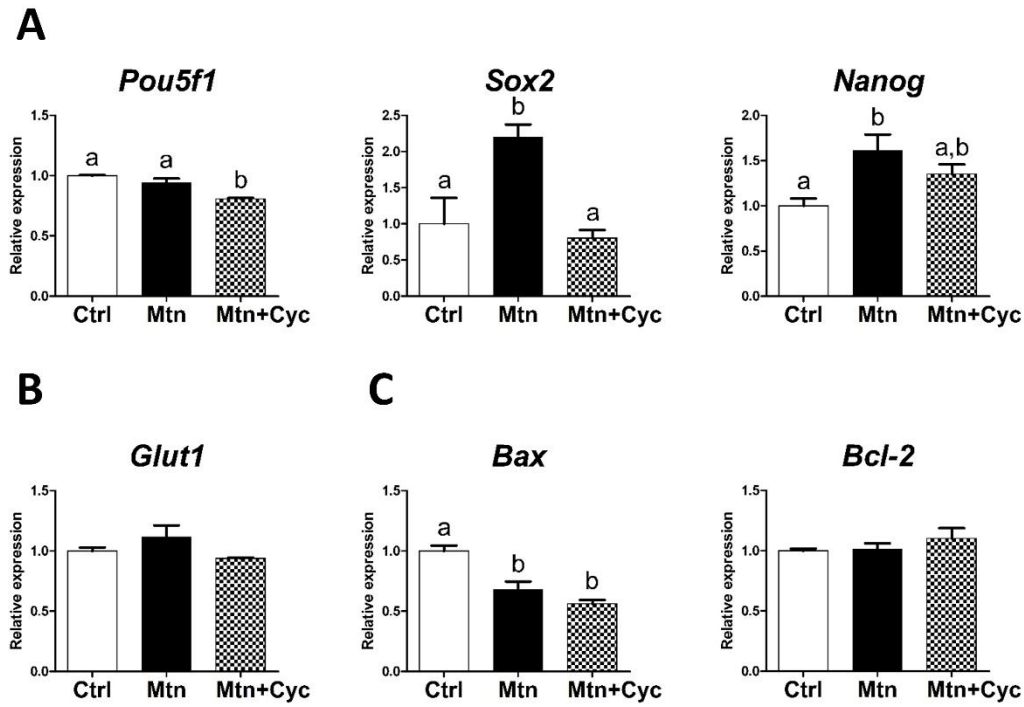


Figure 15. Effect of 10^{-9} M melatonin with or without 2 μM cyclopamine on gene expression in blastocysts derived from parthenogenetically activated oocytes. (A) Developmental competence-related genes (*Pou5f1*, *Sox2* and *Nanog*). (B) Glucose metabolism-related gene (*Glut1*). (C) Apoptosis-related genes (*Bax* and *Bcl-2*). Data are shown as the means ± SEM. Within each category, groups marked with different letters are significantly different ($P < 0.05$). The experiment was replicated at least three times. Ctrl, control; Mtn, 10^{-9} M melatonin; Cyc, 2 μM cyclopamine.

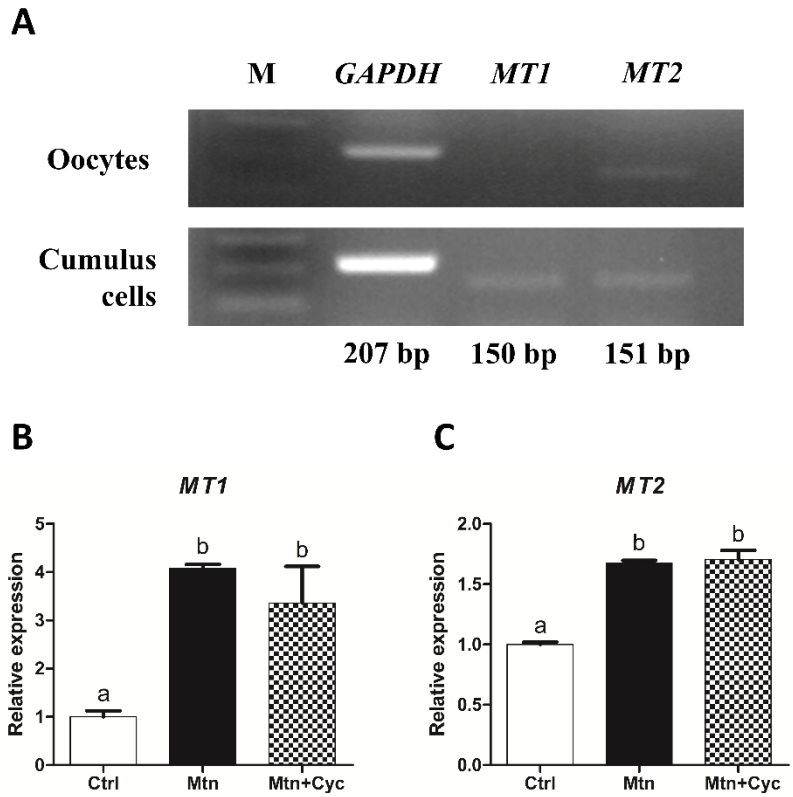


Figure 16. Effect of 10^{-9} M melatonin with or without 2 μM cyclopamine on expression of melatonin receptor genes in cumulus cells. (A) Expression of the transcripts of melatonin receptors (*MT1* and *MT2*) in cumulus cells and oocytes. For each sample, *GAPDH* was amplified to confirm the presence of cDNAs in the reaction. M= a 1 kb marker. (B and C) Effect of 10^{-9} M melatonin with or without 2 μM cyclopamine on expression of melatonin receptor genes (*MT1* and *MT2*) in cumulus cells. Data are shown as the means ± SEM. Within each category, groups marked with different letters are significantly different ($P < 0.05$). The experiment was replicated at least three times. Ctrl, control; Mtn, 10^{-9} M melatonin; Cyc, 2 μM cyclopamine.

4. Discussion

In the present study, for the first time, I demonstrated that a melatonin-induced increase in cumulus expansion, expression of Shh signaling genes and proteins in cumulus cells and embryo development was inhibited by a Shh signaling inhibitor. These results suggest that the ability of melatonin to promote cumulus expansion and subsequent embryo development is at least partially through the activation of Shh signaling in cumulus cells.

Although previously the actions of melatonin have been limited to the brain, subsequent studies have revealed that its receptors exist in numerous tissues outside the nervous system, including the cardiovascular system [194], immune cells [195], retina [196], gastrointestinal tract [197], kidney [198], prostate [199], skin [200] and ovary [115]. In addition to multiple actions mediated by its receptors, melatonin could also directly detoxify reactive oxygen and nitrogen species by its indirect antioxidant properties [201]. Melatonin is a multitasking molecule with numerous receptor-dependent and receptor-independent actions [202]. Although the effects of melatonin on the female reproductive system may or may not be receptor-mediated, melatonin directly influences ovarian function. This could be supported by the existence of melatonin receptors in the ovary [203] and the presence of melatonin in human preovulatory follicular fluid in concentrations that are almost three times higher than serum levels [204]. Moreover, a previous study reported direct effects of melatonin on murine folliculogenesis *in vitro* [205]. For these reasons, melatonin is thought to be effective for improving ovarian function and oocyte quality [113].

Melatonin has been used in various species to improve maturation of oocytes *in vitro* [118, 121, 123]. Particularly, the greatest number of studies were investigated with porcine IVM. Kang et al. [92] reported that melatonin treatment during porcine IVM resulted in significantly increased polar body extrusion and subsequent PA embryo development. In addition, Shi et al. [123] reported the existence of melatonin in porcine follicular fluid and the beneficial effect of melatonin on subsequent embryo development of porcine oocytes treated with melatonin during IVM. Although previous studies have suggested a clear benefit of melatonin in improving porcine IVM, the underlying mechanism by which melatonin directly acts on porcine COCs has not been fully elucidated.

Regarding the physiology of oocyte maturation *in vivo*, the regulation of oocyte meiotic maturation and growth of oocytes are influenced by follicular somatic cell compartments such as the granulosa and cumulus cells surrounding the oocytes. These events are mediated by gap junctions between these cells and the oocyte [206]. Expansion of the granulosa and cumulus cell layers, which is closely related to Hh signaling [132], affects the maturation of oocytes [176]. With this in mind, I hypothesized that melatonin could affect signaling pathways that drive expansion of cumulus cells *in vitro*.

Optimum expansion of the cumulus layer is required for proper maturation of oocytes [177]. In addition, higher rates of fertilization and embryo development have been attributed to expansion of the COCs [179], and cumulus expansion has been used as a gross indicator of oocyte maturation [178]. In the current study, supplementation of

melatonin to the IVM medium significantly increased cumulus expansion of porcine COCs and expression of genes related to cumulus expansion (*Ptgs1*, *Ptgs2*, *Has2*, *Ptx3* and *Tnfaip6*) in cumulus cells. However, cyclopamine significantly blocked these increases in terms of the degree of cumulus expansion and some cumulus expansion genes (*Ptgs1*, *Ptgs2* and *Has2*). To clarify the relationship between the beneficial effects of melatonin on cumulus expansion and Shh signaling, expression of Shh signaling genes and proteins in cumulus cells was also evaluated. Melatonin-induced increases in the expression of Shh signaling genes (*Shh*, *Ptch1*, *Smo* and *Gli1*) and proteins (Ptch1, Smo and Gli1) were inhibited by cyclopamine. Based on these results, it is rational to assume that the Shh signaling pathway mediated the effect of melatonin on cumulus expansion of porcine COCs.

With respect to a possible direct action of melatonin on porcine oocytes, melatonin did not affect nuclear maturation. The meiotic cell cycle progression is controlled by MPF and MAPK-mediated phosphorylation and dephosphorylation [182]. Although melatonin showed significantly increased expression of genes related to oocyte competence (*Gdf9* and *Bmp15*) and the MAPK pathway (*C-mos*) in oocytes, it did not increase MPF pathway-related genes (*Cyclinb1* and *Cdc2*) coding for the principal regulatory molecules that drive meiotic progression [207]. Moreover, although melatonin significantly increased the expression of a Shh signaling gene (*Gli1*) and proteins (Ptch1, Smo and Gli1), it did not increase the expression of some Shh signaling genes (*Shh*, *Ptch1* and *Smo*) which was significantly increased in cumulus cells. Taken

together, these results showed that oocytes were not much influenced by melatonin compared to cumulus cells.

Considering that melatonin showed a greater effect on cumulus cells than on oocytes, this could be explained by the presence of both melatonin receptors (MT1 and MT2) in cumulus cells in contrast to oocytes which have only one melatonin receptor (MT2). In porcine and human follicular fluids, melatonin naturally exists [123, 204]. In addition, binding of a melatonin receptor agonist on the melatonin receptors present in the human ovary has been demonstrated [208]. The presence of melatonin in preovulatory follicular fluid and the existence of its receptors in ovarian follicles imply that melatonin and its receptors have an important role in ovarian and reproductive function [116]. In the present study, *MT2* was observed in both cumulus cells and oocytes. However, *MT1* was only found in cumulus cells. In this regard, the presence of both melatonin receptors (*MT1* and *MT2*) in cumulus cells could explain why melatonin affected cumulus cells more than oocytes.

Oocyte quality is a key determinant of the final outcome of *in vitro* embryo production [209]. Therefore, favorable IVM environment made by melatonin supplementation may contribute to improvements in subsequent embryonic development. In the present study, supplementation of melatonin to the IVM medium significantly improved blastocyst formation rates and total cell numbers. Furthermore, expression of developmental competence genes (*Sox2* and *Nanog*) was significantly increased by melatonin supplementation during IVM. However, concomitant addition of cyclopamine inhibited improvements in blastocyst formation rates, total cell numbers and the

expression of *Sox2*. Based on these results, the beneficial effect of melatonin on subsequent embryo development was abolished when cyclopamine was supplemented simultaneously, indicating that melatonin exerts these beneficial effects through Shh signaling. However, it may not be the only mechanism by which melatonin influences porcine COCs. The expression of a proapoptotic gene (*Bax*) was significantly downregulated by melatonin regardless of the addition of cyclopamine, indicating the possibility that melatonin exerts beneficial actions through antioxidant activities, as previously reported. Nevertheless, our results demonstrate that melatonin enhanced cumulus expansion and subsequent embryo development and that the Shh signaling pathway mediates these processes.

In conclusion, the present study provides the first evidence that the Shh signaling pathway mediates the beneficial effect of melatonin on porcine cumulus expansion. Moreover, melatonin subsequently improved preimplantation development of porcine PA embryos with upregulated embryo development-related genes and a downregulated proapoptotic gene. These findings will be useful for providing insights into the role of Shh signaling in the effect of melatonin on porcine COCs and for investigating potential clinical applications of melatonin. However, further studies are required to study the complete mechanism by which melatonin activates the Shh signaling pathway.

Chapter III. Synergistic effects of resveratrol and melatonin on *in vitro* maturation of porcine oocytes and subsequent embryo development

1. Introduction

Resveratrol (a phytoalexin in grapes) and melatonin (a hormone synthesized by the mammalian pineal gland) are well investigated as natural antioxidants [210] and exhibit many similar biological activities, including free radical scavenging [211], sirtuin-1 activation [212], anti-aging and stem cell protection [213]. For these reasons, there have been attempts to investigate possible synergistic effects of resveratrol and melatonin. Recently, Kwon et al. reported that melatonin synergistically potentiates the neuroprotective effect of resveratrol against oxidative injury by inducing heme oxygenase-1 expression through inhibition of the ubiquitination-dependent proteasome pathway [214]. Moreover, they demonstrated that co-administration of resveratrol and melatonin showed synergistic neuroprotective effects against β-amyloid (Aβ)-induced neuronal death through the AMPK-dependent pathway [215]. In addition to these studies, it was revealed that a combination of resveratrol and melatonin showed chemopreventive effects on rat mammary carcinogenesis induced by N-methyl-N-nitrosourea [216].

Recently, I reported that resveratrol [217] or melatonin [218] has a relationship with the Shh signaling pathway during porcine oocyte IVM. As cumulus expansion is an essential process for oocyte maturation, the fact that resveratrol or melatonin increases porcine cumulus expansion through the activation of Shh signaling suggests a potential benefit of these agents and their possible synergism in porcine IVM. Therefore, I

hypothesized that resveratrol and melatonin could also have synergistic effects on porcine IVM.

Transgenic pigs have been used as animal models for human diseases [219] and IVM is an essential process for producing these animals. This is because handling of pigs in the laboratory and consistently obtaining *in vivo* matured oocytes are difficult compared to other species [41]. However, the developmental competence of porcine oocytes matured *in vitro* is still inferior to that of oocytes matured *in vivo* [220]. Therefore, this study will provide helpful information for improving porcine IVM.

Previous studies demonstrated that resveratrol and melatonin have synergistic effects in pharmacological studies and each individually showed beneficial effects on porcine IVM. However, the possible synergistic effects of these agents on porcine IVM has not been investigated. The aim of this study, therefore, was to investigate the effects of a combination of resveratrol and melatonin on porcine IVM. In the present study, I evaluated the effects of resveratrol, melatonin or their combination on cumulus expansion, oocyte nuclear maturation and subsequent embryonic development.

2. Materials and methods

2.1. Oocyte recovery and IVM

Procedures for IVM were described in general methodology.

2.2. Cumulus expansion assessment

The degree of cumulus expansion was assessed by microscopic examination as described previously [169]. Briefly, a degree of 0 indicates no expansion, characterized by detachment of cumulus cells from the oocyte, leaving a partially or fully denuded oocyte. A degree of 1 indicates the minimum observable response with spherical and compacted cumulus cells around the oocyte. A degree of 2 indicates only the outermost layers of cumulus cells have expanded. A degree of 3 indicates all cell layers except the corona radiata expanded. A degree of 4 indicates the maximum degree of expansion including the corona radiata.

2.3. Assessment of nuclear maturation

After 42-44 h of IVM, COCs were denuded by gently pipetting with 0.1% hyaluronidase in TALP medium and washed three times in TALP medium. The denuded oocytes were evaluated under a microscope (TE2000-S, Nikon Corp.) and classified as immature (without first polar body extrusion), degenerate, or at metaphase-II (MII, with polar body extrusion).

2.4. Parthenogenetic activation of oocytes

Denuded oocytes were gradually equilibrated in activation medium consisting of 0.28 M mannitol, 0.5 mM HEPES, 0.1 mM $CaCl_2$ and 0.1 mM $MgSO_4$ and transferred into a chamber connected to a BTX Electro-Cell Manipulator 2001 (BTX Inc.). Oocytes were activated by a single DC pulse of 1.5 kV/cm for 60 μs. Then, electrically-activated oocytes were washed three times in fresh PZM-5 (Funakoshi Corporation), transferred into wells containing 500 μL PZM-5 and cultured at 39 °C in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2 for 7 days.

2.5. Somatic cell nuclear transfer

Procedures for SCNT were described in the general methodology. In brief, *in vitro* matured oocytes were enucleated by aspirating the first polar body and the adjacent cytoplasm containing chromosomes with an aspiration pipette. Then, using a fine pipette, a trypsinized porcine fetal fibroblast was transferred into the perivitelline space of each enucleated oocyte. These couplets were electrically fused with a single DC pulse of 200 V/mm for 30 μs using an electro cell fusion generator (LF101; Nepa Gene Co.). Then, 30 min after fusion, fused couplets were activated with a single DC pulse of 1.5 kV/cm for 60 μs using a BTX Electro-Cell Manipulator 2001 (BTX Inc.). The resulting activated embryos were cultured in PZM-5 (Funakoshi Corporation) at 39 °C in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2 for 7 days.

2.6. Embryo evaluation and total cell count

The day of PA and SCNT was considered Day 0. Cleavage and blastocyst formation were evaluated on Day 2 (48 h) and on Day 7 (168 h), respectively. To count the total cell numbers of blastocysts, Day 7 blastocysts were washed in TALP medium and then stained with 5 μg/mL Hoechst-33342 for 10 min. After a final wash in TALP medium, stained blastocysts were mounted on glass slides in a drop of 100% glycerol, gently flattened with a cover glass, and observed under a fluorescence microscope (Nikon Corp.) at 400× magnification.

2.7. Gene expression analysis by real-time PCR

Each real-time PCR was performed with isolated mature oocytes and with PA- and SCNT-derived blastocysts. Total RNA was extracted using the easy-spin Total RNA Extraction Kit (iNtRON) and cDNA was synthesized using the RNA to cDNA EcoDry Premix, cDNA synthesis kit (Clontech Laboratories Inc.). The following were placed in a MicroAmp optical 96-well reaction plate (Applied Biosystems): 1 μL cDNA, 0.4 μL (10 pmol/μL) forward primer, 0.4 μL (10 pmol/μL) reverse primer, 10 μL SYBR Premix Ex Taq (Takara, Otsu, Japan), and 8.2 μL of Nuclease-free water (Ambion). The reactions were carried out for 40 cycles and the cycling parameters were as follows: denaturation at 95 ℃ for 15 s, annealing at 60 ℃ for 1 min, and extension at 72 ℃ for 1 min. Primer sequences, expected product sizes and GenBank accession numbers are presented in Table 4. The expression of each target gene was quantified relative to that of the internal control gene (*GAPDH*) using the equation $R = 2^{-[\Delta Ct\ sample - \Delta Ct\ control]}$.

2.8. Statistical analysis

Procedures for statistical analysis were described in general methodology.

Table 4. List of real-time PCR primers

Gene	Primer sequences (5'-3')		Product size (bp)	GenBank accession number
	Forward	Reverse		
GAPDH	GTCGGTTGTGGATCTGACCT	TTGACGAAGTGGTCGTTGAG	207	NM_001206359
Gdf9	CAGTCAGCTGAAGTGGGACA	TGGATGATGTTCTGCACCAT	135	AY626786
Bmp15	CCTCCATCCTTTCCAAGTCA	GTGTAGTACCCGAGGGCAGA	112	NM_001005155
Cyclinb1	CAACTGGTTGGTGTCACTGC	TTCCATCTGCCTGATTTGGT	126	L48205
Cdc2	GGGCACTCCCAATAATGAAGT	GTTCTTGATACAACGTGTGGGAA	260	AB045783
C-mos	GGGAGCAACTGAACTTGGAG	AGAATGTTCGCTGGCTTCAG	115	NM_001113219
Pou5f1	TTTGGGAAGGTGTTCAGCCAAACG	TCGGTTCTCGATACTTGTCCGCTT	198	NM_001113060
Sox2	ATGCACAACTCGGAGATCAG	TATAATCCGGGTGCTCCTTC	130	NM_001123197
Nanog	GGTTTATGGGCCTGAAGAAA	GATCCATGGAGGAAGGAAGA	98	NM_001129971
Glut1	GCTTCCAGTATGTGGAGCAA	AAGCAATCTCATCGAAGGTC	132	XM_013977359
Bax	TGCCTCAGGATGCATCTACC	AAGTAGAAAAGCGCGACCAC	199	XM_003127290
Bcl2	AGGGCATTCAGTGACCTGAC	CGATCCGACTCACCAATACC	193	NM_214285

3. Results

3.1. Effects of resveratrol, melatonin or their combination on cumulus expansion

In the first experiment, all treatment groups including resveratrol, melatonin and their combination significantly increased the proportion of COCs exhibiting complete cumulus expansion (degree 4) and decreased the proportion of degree 3 compared to the control (Fig. 17). Degree 2 expansion was significantly decreased by the resveratrol and combination groups.

3.2. Effects of resveratrol, melatonin or their combination on oocyte nuclear maturation

In experiment 2, the effects of 2 μM resveratrol and/or 10^{-9} M melatonin treatment during IVM on oocyte nuclear maturation was investigated (Fig. 18). Resveratrol significantly increased the metaphase II rate compared to the control (88.9% vs. 84.0%, respectively) and decreased the degeneration rate (3.8% and 6.8%, respectively). However, melatonin treatment showed no significant differences in metaphase II, immature and degeneration rates compared to the control. The combination group showed the highest nuclear maturation rate (93.6%) compared to all other groups (control, 84.0%; resveratrol, 88.9%; melatonin, 84.4%) and significantly decreased the immature rate compared to the control (4.1% vs. 9.2%, respectively) and degeneration rate (2.3% vs. 6.8%, respectively). In addition, I compared the expression of genes related to oocyte

competence (*Gdf9* and *Bmp15*), MPF (*Cyclinb1* and *Cdc2*), and the MAPK pathway (*C-mos*) in oocytes (Fig. 19). Resveratrol and the combination treatment showed significantly increased expression of all investigated genes (*Gdf9*, *Bmp15*, *Cyclinb1*, *Cdc2* and *C-mos*), while melatonin significantly increased expression of *Gdf9*, *Cdc2* and *C-mos* compared to the control. In particular, the combination group showed the highest expression of *Bmp15* and *C-mos*, but the highest expression of *Cdc2* was found in the resveratrol group.

3.3. Effects of resveratrol, melatonin or their combination during IVM on subsequent development of PA embryos

In experiment 3, I evaluated the effects of 2 μM resveratrol and/or 10^{-9} M melatonin treatment during IVM on subsequent embryonic development after PA (Fig. 20). All treatment groups (resveratrol, melatonin and their combination) showed a significant increase in blastocyst formation rates and total cell numbers compared with the control (resveratrol, 34.3% and 56.7; melatonin, 33.9% and 58.1; combination; 34.3% and 65.6; control, 24.3% and 46.9, respectively). In particular, the combination showed the highest total cell numbers compared to all the other groups. In addition, the resveratrol and combination groups significantly increased cleavage rates (90.5% and 90.2%, respectively) compared to the control (82.0%), but no significant increase was observed in the melatonin group (85.3%). Then, expression of genes related to embryo development (*Pou5f1*, *Sox2* and *Nanog*), glucose metabolism (*Glut1*) and apoptosis (*Bax* and *Bcl-2*) was investigated in PA-derived blastocysts (Fig. 21). Blastocysts derived from the

resveratrol group had significantly increased expression of *Pou5f1*, *Sox2*, *Nanog*, *Glut1* and *Bcl-2* compared to the control. The melatonin group showed significantly increased expression of *Nanog* and decreased expression of *Bax* compared with the control group. The combination group exhibited significantly increased *Pou5f1*, *Sox2*, *Nanog*, *Glut1* and *Bcl-2* and decreased expression of *Bax* compared to the control. In particular, expression of *Pou5f1* and *Bcl-2* in the combination group was the highest compared to all the other groups

3.4. Effects of the combination of resveratrol and melatonin during IVM on subsequent development of SCNT embryos

In experiment 4, the effects of combined treatment with 2 μM resveratrol and 10^{-9} M melatonin during IVM on subsequent embryonic development after SCNT was investigated (Fig. 22). The combination of resveratrol and melatonin significantly increased the rate of blastocyst formation and total cell numbers after SCNT compared to the control (23.9% and 43.8 vs. 13.4% and 30.0, respectively). There was no significant difference in cleavage rates. In addition, I investigated the expression of genes related to embryo development, glucose metabolism and apoptosis in SCNT-derived blastocysts (Fig. 23). Blastocysts derived from the combination group showed significantly increased expression of *Pou5f1* and *Glut1* and decreased expression of *Bax* compared to the control.

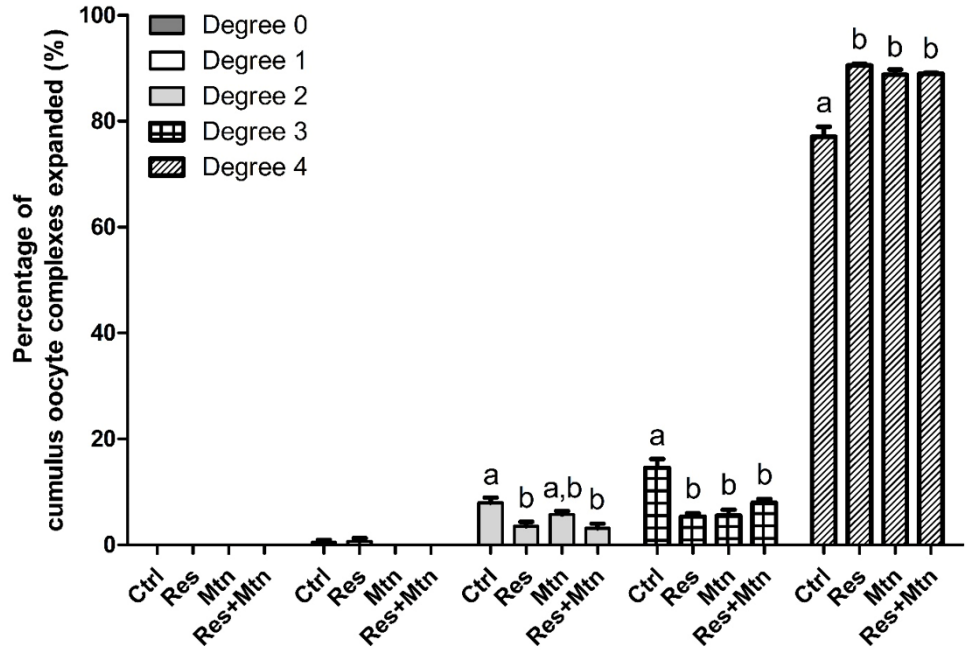


Figure 17. Effects of resveratrol, melatonin or their combination on cumulus expansion at 44 h of IVM. The degree of cumulus expansion was classified into five groups. Data are shown as the means ± SEM. Within each category, groups marked with different letters are significantly different ($P < 0.05$). A total 763 oocytes was used in four independent replicates. Ctrl, control; Res, 2 μM resveratrol; Mtn, 10^{-9} M melatonin.

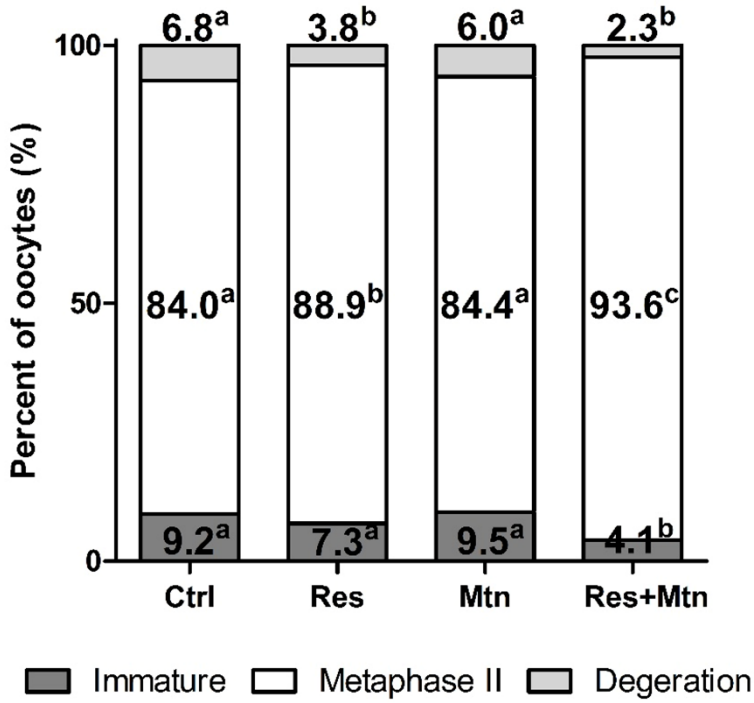


Figure 18. Effects of resveratrol, melatonin or their combination on the nuclear maturation of porcine oocytes. Data are shown as the means ± SEM. Within each category, groups marked with different letters are significantly different ($P < 0.05$). A total 1029 oocytes was used in four independent replicates. Ctrl, control; Res, 2 μM resveratrol; Mtn, 10^{-9} M melatonin.

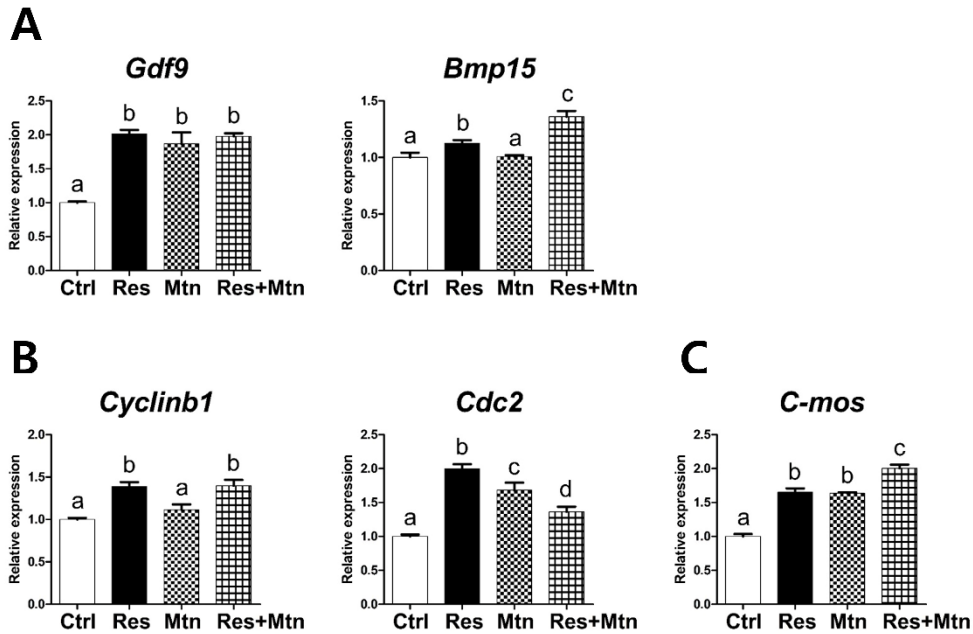


Figure 19. Effects of resveratrol, melatonin or their combination on gene expression in porcine oocytes. (A) Oocyte competence-related genes (*Gdf9* and *Bmp15*). (B) Maturation promoting factor (MPF)-related genes (*Cyclinb1* and *Cdc2*). (C) The mitogen-activated protein kinases (MAPK) pathway-related gene (*C-mos*). Data are shown as the means ± SEM. Within each category, groups marked with different letters are significantly different ($P < 0.05$). The experiment was replicated at least three times. Ctrl, control; Res, 2 μM resveratrol; Mtn, 10^{-9} M melatonin.

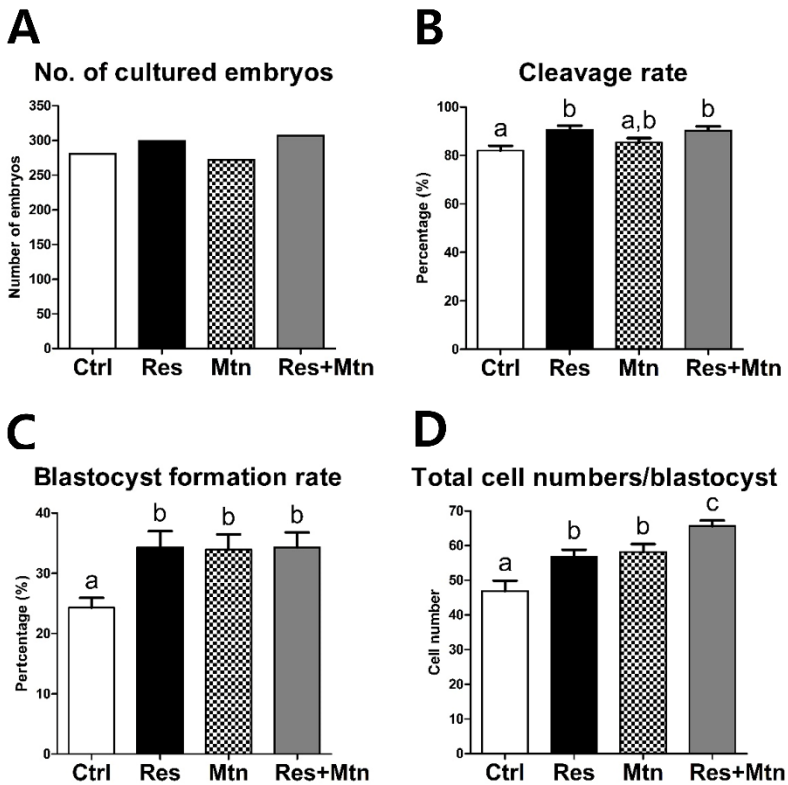


Figure 20. Effects of resveratrol, melatonin or their combination on subsequent embryo development after parthenogenetic activation. (A) Number of cultured embryos. (B) Cleavage rate. (C) Blastocyst rate. (D) Total cell numbers/blastocyst. Data are shown as the means ± SEM. Within each category, groups marked with different letters are significantly different ($P < 0.05$). A total of 1159 oocytes was used in seven independent replicates. Ctrl, control; Res, 2 μM resveratrol; Mtn, 10^{-9} M melatonin.

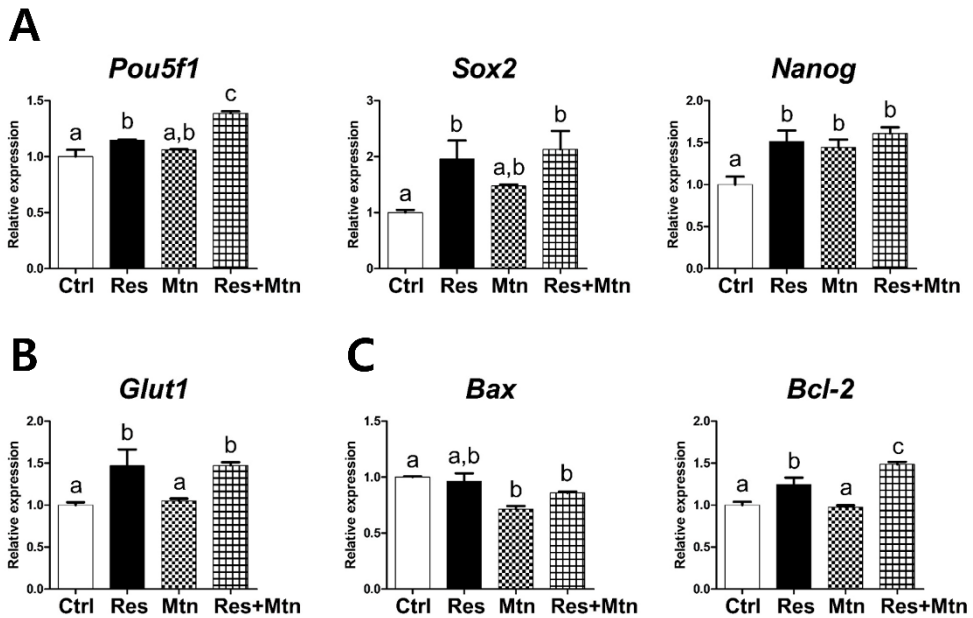


Figure 21. Effects of resveratrol, melatonin or their combination on gene expression in blastocysts derived from parthenogenetically activated oocytes. (A) Developmental competence-related genes (*Pou5f1*, *Sox2* and *Nanog*). (B) Glucose metabolism-related gene (*Glut1*). (C) Apoptosis-related genes (*Bax* and *Bcl-2*). Data are shown as the means ± SEM. Within each category, groups marked with different letters are significantly different ($P < 0.05$). The experiment was replicated at least three times. Ctrl, control; Res, 2 μM resveratrol; Mtn, 10^{-9} M melatonin.

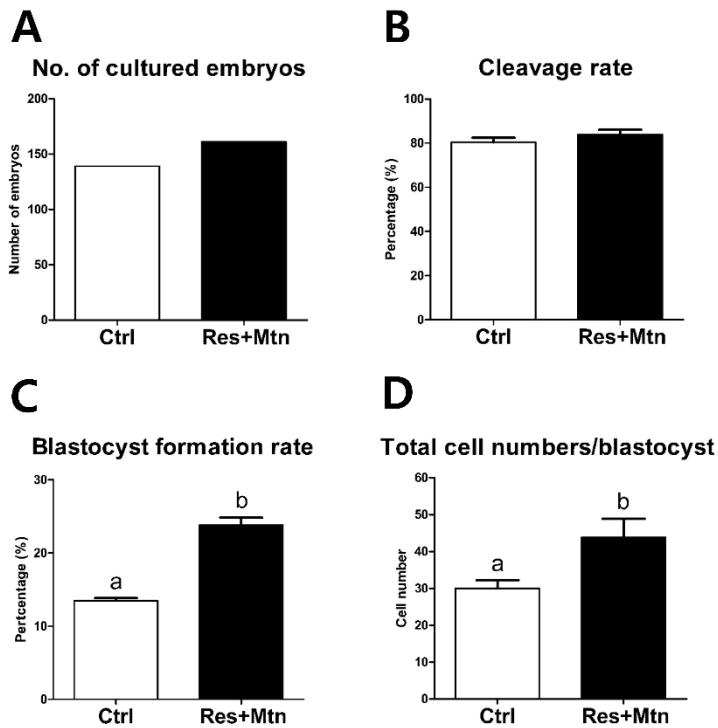


Figure 22. Effects of the combination of resveratrol and melatonin on subsequent embryo development after somatic cell nuclear transfer. (A) Number of cultured embryos. (B) Cleavage rate. (C) Blastocyst rate. (D) Total cell numbers/blastocyst. Data are shown as the means ± SEM. Within each category, groups marked with different letters are significantly different ($P < 0.05$). A total of 300 oocytes was used in six independent replicates. Ctrl, control; Res, 2 μM resveratrol; Mtn, 10^{-9} M melatonin.

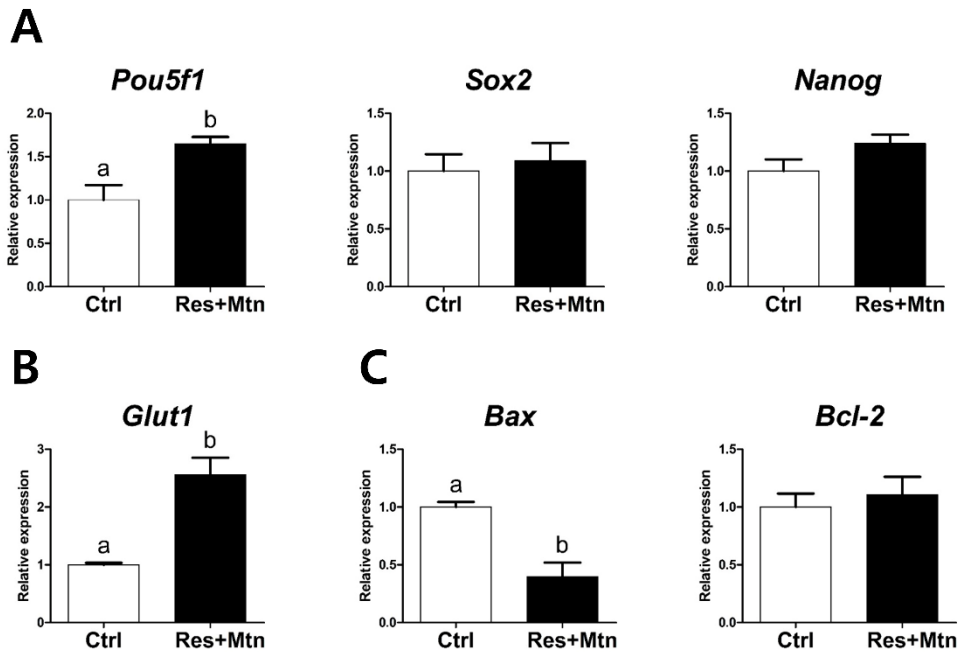


Figure 23. Effects of the combination of resveratrol and melatonin on gene expression in somatic cell nuclear transfer blastocysts. (A) Developmental competence-related genes (*Pou5f1*, *Sox2* and *Nanog*). (B) Glucose metabolism-related gene (*Glut1*). (C) Apoptosis-related genes (*Bax* and *Bcl-2*). Data are shown as the means ± SEM. Within each category, groups marked with different letters are significantly different ($P < 0.05$). The experiment was replicated at least three times. Ctrl, control; Res, 2 μM resveratrol; Mtn, 10^{-9} M melatonin.

4. Discussion

Possible synergistic effects of resveratrol and melatonin have been suggested by previous studies [214-216]. However, effects of a combination of these agents on porcine IVM were not investigated. In this report, I demonstrated that the supplementation of 2 μM resveratrol and 10^{-9} M melatonin to the IVM culture medium has synergistic effects on both oocyte nuclear maturation and the total cell numbers of PA-derived blastocysts compared to resveratrol or melatonin alone. Furthermore, the combination of these chemicals during IVM improved the efficiency of SCNT in terms of blastocyst formation rates and total cell numbers.

During oogenesis, cumulus cell expansion plays an important role in oocyte maturation [176] and this is closely correlated with developmental competence of oocyte [221]. Therefore, I evaluated cumulus expansion, oocyte nuclear maturation and subsequent embryo development to investigate the possible synergistic effects of resveratrol and melatonin on IVM.

Cumulus expansion has been used as a criterion of oocyte quality following maturation [222]. This is because optimum expansion of the cumulus cell layers is required for proper maturation of oocytes [177] and therefore, increased rates of subsequent embryo development are attributed to cumulus expansion [179]. Cumulus expansion of porcine COCs was significantly increased by all the treatment groups, resveratrol, melatonin or their combination. Although the combination treatment did not show more beneficial effects on cumulus expansion

than resveratrol or melatonin alone, it also significantly improved cumulus expansion compared to the control.

Regarding the effects of resveratrol, melatonin or their combination on oocyte nuclear maturation, resveratrol and the combination treatment showed significantly increased MII rates. Moreover, they significantly increased the expression of all investigated genes related to oocyte competence (*Gdf9* and *Bmp15*), MPF (*Cyclinb1* and *Cdc2*) and the MAPK pathway (*C-mos*) in oocytes. Particularly, the combination group showed the highest MII rates and expression of *Bmp15* and *C-mos* compared to all other groups. The synergistic effect of the combination treatment on nuclear maturation might be due to its highest expression of *C-mos*. Mos (the *C-mos* proto-oncogene product) mediates the activity of MPF, another essential regulator of meiosis resumption formed by cyclin B1 and Cdc2 kinase [223]. These results suggested that the combination of resveratrol and melatonin was the optimal IVM supplement for porcine oocyte nuclear maturation.

When oocytes matured with resveratrol, melatonin or their combination were parthenogenetically activated, the results showed that the combination treatment appeared to be better than resveratrol or melatonin alone because it supported the highest total cell numbers of blastocysts. Although either resveratrol or melatonin could also increase blastocyst formation and total cell numbers, the increased total cell numbers were significantly lower than that of the combination treatment. Because the total cell numbers of blastocysts is an indication for evaluating embryo quality [224], this result indicates that the combination of resveratrol and

melatonin had a synergistic effect on embryo quality. This synergistic effect was reflected in gene expression of SCNT blastocysts. The combination treatment had significantly higher expression of genes related to embryo development (*Pou5f1*) and anti-apoptosis (*Bcl-2*) compared to resveratrol or melatonin alone. Because *Pou5f1* is a marker for early embryo development [225] and Bcl-2 regulates early embryo survival by involving the apoptotic response [226], an increase in expression of these genes by the combination treatment supported the results showing a synergistic improvement in embryo quality. Therefore, the combination of resveratrol and melatonin was selected as the optimal supplement for porcine IVM.

Based on these results, SCNT was performed using oocytes matured with the combination of resveratrol and melatonin. The combination significantly improved blastocyst formation and total cell numbers of SCNT embryos, indicating improvements in preimplantation development and the quality of cloned embryos. In gene expression analysis using SCNT blastocysts, the combination group showed significantly increased expression of *Pou5f1* and *Glut1* and decreased expression of *Bax* compared to the control. The difference in gene expression pattern between PA and SCNT blastocysts was probably due to a different type of nuclear reprogramming [104, 227]. On the basis of these SCNT results, I concluded that the combination treatment of resveratrol and melatonin during IVM supported the development of porcine cloned embryos.

In conclusion, the present study suggests that the combination of resveratrol and melatonin has synergistic effects on porcine IVM and could be applied to

production of high quality porcine blastocysts. These findings will be useful for *in vitro* embryo production technologies and potential clinical applications.

PART IV

PRODUCTION OF CLONED EMBRYOS USING *KLOTHO* GENE MODIFIED PORCINE FIBROBLASTS

Chapter I. Production of cloned embryos using *klotho*-knockout porcine fibroblasts

1. Introduction

Genetically engineered pigs are useful models for studying human diseases, because of the similarity of their anatomy and physiology to those of humans [228]. Recent advances in genome editing techniques such as Zinc-Finger nucleases (ZFNs), Transcription activator-like effector nucleases (TALENs) and the Clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas9) system have enabled the production of animal models for specific purposes [229]. In particular, recent application of the CRISPR/Cas9 system improved the efficiency of genome editing with higher percentage of desired mutation compared to previous meganucleases (ZFNs and TALENs) [230].

CRISPR-mediated pig genome editing is typically accomplished by SCNT with donor cells transfected with single-guide RNA (sgRNA) and Cas9 DNA to generate modified animals [231]. However, transfection with plasmid DNA encoding sgRNA and Cas9 is limited by off-target effects and unwanted integration of DNA segments at both on-target and off-target sites in the genome [232]. To overcome this, nowadays, delivery of Cas9-sgRNA ribonuleoproteins (RNPs) into cells or embryos was frequently used. Furthermore, delivery of pre-assembled Cas9-sgRNA RNPs facilitates highly efficient genome editing in cells, embryos and organisms [233-235].

The klotho deficient mice display multiple aging-like phenotypes similar to human premature-aging syndromes due to a defect in *klotho* gene expression [141]. They develop normally until 3 weeks of age, but afterward they begin to show multiple aging-like phenotypes such as growth retardation, infertility, arteriosclerosis, osteoporosis and finally premature death at 2 months of age.

Although *klotho* knockout mice exhibits multiple aging-like phenotypes, pigs could be more suitable animal models for human aging because of the similarity of their anatomy and physiology to those of humans [219]. However, studies using large animal models such as pigs have been limited due to the absence of cell lines or animal models. Therefore, the objective of this study was 1) to determine whether the use of non-selected donor cells transfected with Cas9-sgRNA RNPs for SCNT results in high mutation rates in embryos and fetuses for establishment of *klotho* knockout fetal cell lines and 2) to generate preimplantation embryos cloned from this cell lines in order to produce *klotho* knockout pigs.

2. Materials and methods

2.1. Animals

The animals used in this study were maintained by the R&F farm (Boryeong, Korea) and the research farm of Gyeonggi Livestock and Veterinary Service (Osan, Korea). All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of Seoul National University (SNU-160613-16) in accordance with the Guide for the Care and Use of Laboratory Animals of Seoul National University.

2.2 Design and construction of *klotho* targeting CRISPR/Cas9 system

The sgRNA that could recognize porcine *klotho* gene were designed using a CRISPR RGEN Tools (http://www.rgenome.net). Sequence information of the designed sgRNA is 5′-TAGAACAAGGCTGAAGACTTCGG-3′. The protospacer adjacent motif (PAM) and sgRNA targeting sequence can be identified by the blue and red fonts, respectively (Fig. 24). Specificity of the designed sgRNA was confirmed by searching for similar porcine sequences in GenBank. The sgRNA was designed to create double strand breaks (DSBs) in exon 3 of *klotho*.

2.3. Delivery of Cas9-sgRNA ribonucleoproteins

To introduce DSBs in wild type porcine fetal fibroblasts using RNPs, $9x10^5$ cells were transfected with Cas9 protein (28.8 μg) premixed with *in vitro*

transcribed sgRNA (7.2 μg) through nucleofection (Neon; Invitrogen) with a 1400 V, 30 ms pulse width, and pulse number 1 setting. Transfected cells were subjected to a 4-well cell culture dish. After 1-2 days of transfection, non-selected cell population was directly used for SCNT. Cas9 protein in storage buffer (20 mM HEPES pH 7.5, 150 mM KCl, 1 mM DTT, and 10% glycerol) was mixed with sgRNA dissolved in nuclease-free water and incubated for 10 min at room temperature before use.

2.4. Somatic cell nuclear transfer

Procedures for SCNT were described in general methodology. The combination of 2 μM resveratrol and 10^{-9} M melatonin was supplemented to the IVM medium during the entire maturation period, when we performed SCNT for embryo transfer.

2.5. Embryo transfer, pregnancy test and fetus recovery

Procedures for embryo transfer and pregnancy test were described in general methodology. In brief, one- to four-cell stage SCNT embryos were transferred into a naturally cycling recipient pig on day two after standing estrus was observed. A midventral laparotomy was performed under general anesthesia using isoflurane. The reproductive tract was exposed and the SCNT embryos (150– 250 embryos) were transferred into both oviducts of a recipient pig. Pregnancy was diagnosed by ultrasonography on Day 25 (The day of SCNT was considered Day 0). If the surrogate became pregnant, the gestation was monitored every two weeks. If

needed, fetuses were recovered on Day 28 post transfer.

2.6. Primary culture of porcine fetal fibroblasts

Fetal fibroblasts from 7 cloned porcine fetuses were isolated and cultured. Procedures for Primary culture of porcine fetal fibroblasts were described in general methodology.

2.7. T7E1 assay

Genomic DNA was extracted using Exgene TM cell SV (GeneAll Biotech., Seoul, Korea), according to the manufacturer's instructions. The genomic flanks containing CRISPR/Cas9 target sites were amplified using the primers listed in Table 5. The T7E1 analysis was conducted as described previously [236]. Briefly, the PCR amplicons were denatured at 95 °C and annealed to form heteroduplex DNA, which were digested with 5 units of T7 endonuclease 1 (ToolGen Inc., Seoul) for 20 min at 37 °C and then analyzed by 2% agarose gel electrophoresis.

2.8. Deep sequencing

The on-target regions were amplified from genomic DNA and used for library construction. Equal amounts of the PCR amplicons were subjected to paired-end read sequencing using Illumina MiSeq (v2, 300-cycle). Rare sequence reads that occur only once were excluded to remove errors associated with sequencing reaction and amplification. Insertions or deletions located around the

CRISPR/Cas9 cleavage site (3 bp upstream of the PAM) were considered to be the mutations induced by CRISPR/Cas9.

2.9. Gene expression analysis by real-time PCR

All samples were stored at -80°C until analysis. Total RNA was extracted using the easy-spin Total RNA Extraction Kit (iNtRON), according to the manufacturer's protocol, and the total RNA concentration was quantified using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). The cDNA was synthesized using Maxime RT Premix (iNtRON) according to the manufacturer's protocol. A PCR plate (MicroAmp optical 96-well reaction plate) was made by adding 1 μL cDNA, 0.4 μL (10 pmol/μL) forward primer, 0.4 μL (10 pmol/μL) reverse primer, 10 μL SYBR Premix Ex Taq (Takara) and 8.2 μL of Nuclease-free water (Ambion). The reactions were carried out for 40 cycles and the cycling parameters were as follows: denaturation at 95°C for 15 s, annealing at 60°C for 1 min and extension at 72°C for 1 min. All oligonucleotide primer sequences are presented in Table 6. The expression of each target gene was quantified relative to that of the internal control gene (*GAPDH*) using the equation, $R = 2^{-[\Delta Ct\ sample - \Delta Ct\ control]}$.

2.10. Statistical analysis

Procedures for statistical analysis were described in general methodology.

Table 5. Details of primers used for T7E1 assay and deep sequencing

Target	Use	Primer sequences (5'-3')	Product size (bp)
Klotho	T7E1	F: CCTCAAGTAGTAAAACCCTC R: GGTTTTGTCAGCTGACTTAC	379
	Deep sequencing	F: CTTGCTCTTGTCCTCTTTCC R: CAACAATTCCCCAAGCAAAG	282

Table 6. List of real-time PCR primers

Gene	Primer sequences (5'-3')		Product size (bp)	GenBank accession number
	Forward	Reverse		
GAPDH	GTCGGTTGTGGATCTGACCT	TTGACGAAGTGGTCGTTGAG	207	NM_001206359
IGF1	AGGAGGCTGGAGATGTACTG	TGGCATGTCATTCTTCACTC	191	NM_214256
IGF1R	ATTCGCACCAATGCTTCA	AGGGCGGGTTCCACTTC	94	NM_214172
FOXO1	CATTGAGCGCTTAGACTGTG	TCTCAGTTCCTGCTGTCAGA	214	NM_214014
MnSOD	GCTTACAGATTGCTGCTTGT	AAGGTAATAAGCATGCTCCC	101	NM_214127
CAT	TTAATCCATTCGATCTCACC	GGCGGTGAGTGTCAGGATAG	210	NM_214301
Caspase3	CGTGCTTCTAAGCCATGGTG	GTCCCACTGTCCGTCTCAAT	186	NM_214131
Bax	TGCCTCAGGATGCATCTACC	AAGTAGAAAAGCGCGACCAC	199	XM_003127290
Bcl-2	AGGGCATTCAGTGACCTGAC	CGATCCGACTCACCAATACC	193	NM_214285

3. Results

3.1. Evaluation of embryo development and genome editing efficiency after SCNT using non-selected donor cells transfected with *klotho* targeting Cas9-sgRNA RNPs

I compared the preimplantation development of embryos after SCNT with Cas9-sgRNA RNPs (targeting the *klotho* gene) transfected or non-transfected fibroblasts (Table 7). I made a total of 253 SCNT embryos using non-transfected donor cells (n=58) and transfected donor cells (n=195). Some of them developed into blastocysts (n=6 and 20, respectively) and there was no significant difference in blastocyst formation rates (8.1% vs. 11.3%). However, there was a significant difference in fusion rates between non-transfected and transfected groups (95.5% vs. 89.4%). In T7E1 assay, thirteen of twenty (65.0%) blastocysts derived from Cas9-sgRNA RNPs transfected cells showed modifications in the *klotho* gene (Fig. 25A). To confirm the modifications, I performed deep sequencing analysis and the results showed that eight blastocysts contained monoallelic modifications (40.0%) and five blastocysts contained biallelic modifications (25.0%) (Table 8 and Fig. 25B).

3.2. Evaluation of genome editing efficiency after embryo transfer and establishment of *klotho* knockout fetal cell lines

Based on high modification rates in preimplantation embryos, I performed embryo transfer after SCNT with non-selected donor cells transfected with *klotho*

targeting Cas9-sgRNA RNPs. I transferred a total of 936 SCNT embryos to five recipients and one recipient became pregnant (Table 9). After 28 days, sixteen cloned fetuses were recovered. Nine of them were absorbed and seven of them were living (Fig. 26). I performed primary culture using body parts of seven living fetuses to establish *klotho* knockout fetal cell lines and carried out deep sequencing using the remaining tissues of living and absorbed fetuses. In deep sequencing analysis, four (44.4%) of nine absorbed fetuses and three (42.9%) of seven living fetuses showed monoallelic modifications (Table 10 and Fig. 27).

3.3. Effect of *klotho* monoallelic knockout on gene expression in fetal fibroblasts

Gene expression in *klotho* monoallelic knockout fetal fibroblasts (Fetus L2) and wild type fetal fibroblasts was compared. In this experiment, expression of genes related to aging (IGF1 signaling genes, FOXO1 and antioxidant genes) and apoptosis was evaluated. As shown in Figure 28, expression of *IGF1* and *IGF1R* was significantly decreased in the Fetus L2 fibroblasts. In addition, Fetus L2 showed significantly decreased expression of *FOXO1* and its downstream target genes with antioxidant function (*MnSOD* and *CAT*). Regarding apoptosis-related genes, the *Bax*/*Bcl2* ratio and expression of *Caspase3* were significantly increased in Fetus L2 fibroblasts.

3.4. Preimplantation development of cloned embryos derived from *klotho* monoallelic knockout fetal fibroblasts.

As shown in Table 11, no significant difference was observed in cleavage rates and blastocysts formation rates between cloned embryos derived from wild type and Fetus L2 fibroblasts (82.8% and 15.2% vs. 85.5% and 14.3%, respectively).

3.5. Results of transfer of SCNT embryos cloned from *klotho* monoallelic knockout porcine fetal fibroblasts.

As shown in Table 12, a total of 2088 SCNT embryos cloned from *klotho* monoallelic knockout porcine fibroblasts (Fetus L2 and L3) were transferred to eleven synchronized recipients. Seven from eleven recipients (63.6%) became pregnant. However, all of them were aborted.

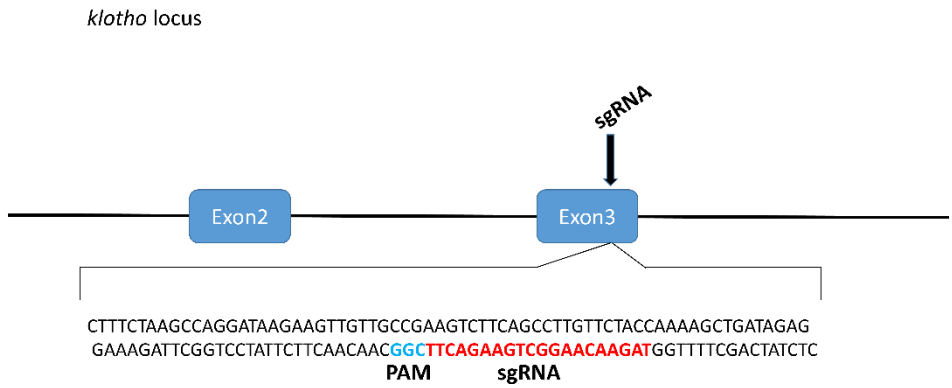


Figure 24. Schematic representation of sgRNA specific to exon 3 of the porcine *klotho* locus. The sgRNA targeting sequence is highlighted in red and protospacer adjacent motif (PAM) is highlighted in blue.

Table 7. Comparison of preimplantation development of porcine embryos after SCNT with Cas9-sgRNA RNPs (targeting the *klotho* gene) transfected or non-transfected fibroblasts

NT groups	No. oocytes		No. of embryos developed to (%)		Mutated blastocysts (%)
	Injected	Fused (%)*	≥ 2-cells**	Blastocyst***	
Non-transfected	61	58 (95.5 ± 1.1)[a]	46 (81.4 ± 4.0)	6 (8.1 ± 2.2)	-
Transfected	219	195 (89.4 ± 1.6)[b]	149 (76.8 ± 3.6)	20 (11.3 ± 3.1)	13/20 (65.0)

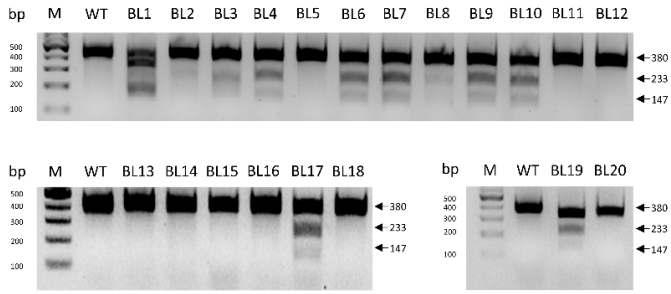
Values with different superscript letters within a column are significantly different ($P < 0.05$). Experiment was replicated at least three times. The data represent means ± SEM.

* Percentage of the number of injected oocytes.

** Percentage of the number of fused oocytes undergoing cleavage.

*** Percentage of the number of fused oocytes developed into blastocysts.

A



B

Wild type genomic DNA		
	CTTTCTAAGCCAGGATAAGAAGTTGTTGCCGAAGTCTTCAGCCTTGTTCTACCAAAAGCTGATAGAG	
Monoallelic modifications		
BL3	CT----------TCAGCCTGT----------TCTTCAGCCTTGTTCTACCAAAAGCTGATAGAG	**-32bp,+10bp**
BL4	CTTTCTAAGCCAGGATAAGAAGTTGTT----------GCCTTGTTCTACCAAAAGCTGATAGAG	**-13bp**
BL6	CTTTCTAAGCCAGGATAAGAAGTTGTT----------GCCTTGTTCTACCAAAAGCTGATAGAG	**-13bp**
BL7	CTTTCTAAGCCAGGATAAGAAGTTGTT----------GCCTTGTTCTACCAAAAGCTGATAGAG	**-13bp**
BL8	CTTTCTAAGCCAGGATAAGAAGTTGTTGCCGAA--CTTCAGCCTTGTTCTACCAAAAGCTGATAGAG	**-2bp**
BL9	CTTTCTAAGCCAGGATAAGAAGTTGTT----------GCCTTGTTCTACCAAAAGCTGATAGAG	**-13bp**
BL16	CTTTCTAAGCCAGGATAAGAAGTTGTTGCCGAAGTTCTTCAGCCTTGTTCTACCAAAAGCTGATAGAG	**+1bp**
BL19	CTTTCTAAGCCAGGATAAGAAGTTGTT----------GCCTTGTTCTACCAAAAGCTGATAGAG	**-13bp**
Biallelic modifications		
BL1	CTTTCTAAGCCAGGATAAGAAGTTGTT----------GCCTTGTTCTACCAAAAGCTGATAGAG	**-13bp**
BL2	CTTTCTAAGCCAGGATAAGAAGTTGTTG------CTT------TTGTTCTACCAAAAGCTGATAGAG	**-15bp,+3bp**
	CTTTCTAAGCCAGGATAAGAAGTTGTTGCCGAAGTTCTTCAGCCTTGTTCTACCAAAAGCTGATAGAG	**+1bp**
BL10	CTTTCTAAGCCAGGATAAGAAGTTGTT----------GCCTTGTTCTACCAAAAGCTGATAGAG	**-13bp**
	CTTTCTAAGCCAGGATAAGAAGTTGTTGCC----------GCCTTGTTCTACCAAAAGCTGATAGAG	**-10bp**
BL15	CTTTCTAAGCCAGGATAAGAAGTTGTTGCCGAAG-CTTCAGCCTTGTTCTACCAAAAGCTGATAGAG	**-1bp**
BL17	CTTTCTAAGCCAGGATAAGAAGTTGTTGCCGAA----------GTTCTACCAAAAGCTGATAGAG	**-12bp**

Figure 25. Generation of *klotho* gene knockout blastocysts by SCNT using non-selected porcine fibroblasts transfected with Cas9-sgRNA RNPs. (A) T7 endonuclease I (T7E1) assay: the T7E1 assay was conducted using genomic DNA from twenty cloned blastocysts. (M, Marker; WT, wild type; BL, blastocyst). (B) A diagram illustrating the editing scheme on exon 3 of the *klotho* gene of blastocysts.

Table 8. Rate of DNA editing on the *klotho* gene of blastocysts generated by SCNT using non-selected porcine fibroblasts transfected with Cas9-sgRNA RNPs

No. of SCNT blastocysts	Type of editing events detected by deep sequencing		
	Unedited	Monoallelic	Biallelic
20	7 (35.0%)	8 (40.0%)	5 (25.0%)

Table 9. Results of transfer of SCNT embryos cloned from non-selected porcine fibroblasts transfected with Cas9-sgRNA RNPs (targeting the *klotho* gene)

Recipient	No. of transferred cloned embryos	Pregnancy	No. of recovered fetuses	Absorbed	Living
1	173	No	-	-	-
2	240	No	-	-	-
3	163	No	-	-	-
4	178	No	-	-	-
5	182	Yes	16	9	7

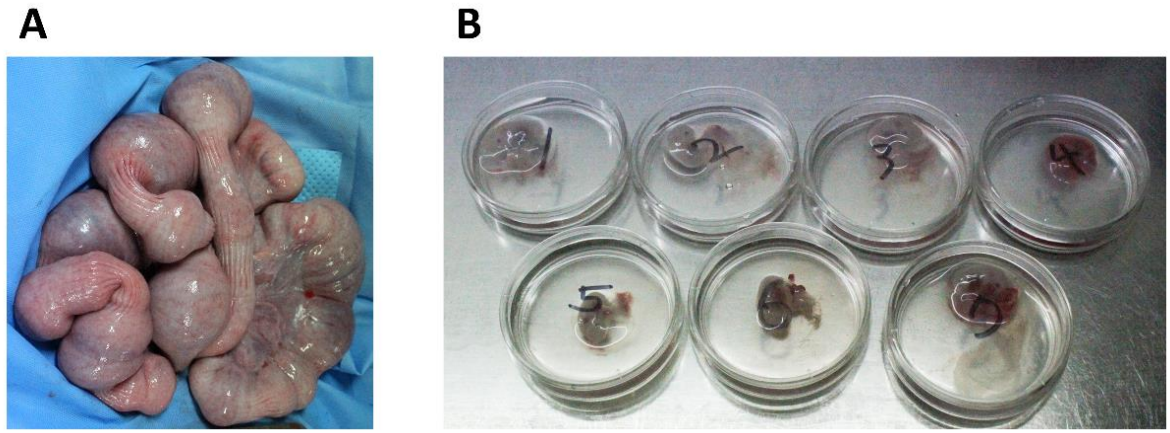


Figure 26. The uterus of the recipient (A) and the living fetuses (B) at day 28 of gestation.

Wild type genomic DNA		
	CTTTCTAAGCCAGGATAAGAAGTTGTTGCCGAAGTCTTCAGCCTTGTTCTACCAAAAGCTGATAGAG	
Monoallelic modifications		
Fetus A4	CTTTCTAAGCCAGGATAAGAAGTTGTTGCCGAAG-CTTCAGCCTTGTTCTACCAAAAGCTGATAGAG	**-1bp**
Fetus A5	CTTTCTAAGCCAGGATAAGAAGTTGTT----------GCCTTGTTCTACCAAAAGCTGATAGAG	**-13bp**
Fetus A6	CTTTCTAAGCCAGGATAAGAAGTTGTTGCCGAAG--TTCAGCCTTGTTCTACCAAAAGCTGATAGAG	**-2bp**
Fetus A9	CTTTCTAAGCCAGGATAAGAAGTTGTTGCC----------GATAGAG	**-30bp**
Fetus L1	CTTTCTAAGCCAGGATAAGAAGTTGTTGC------CTTCAGCCTTGTTCTACCAAAAGCTGATAGAG	**-6bp**
Fetus L2	CTTTCTAAGCCAGGATAAGAAGTTGTTGC--**AACTTCTTACCA**---TTCTACCAAAAGCTGATAGAG	**-17bp,+12bp**
Fetus L3	CTTTCTAAGCCAGGATAAGAAGTTGTTGCCGAAG-CTTCAGCCTTGTTCTACCAAAAGCTGATAGAG	**-1bp**

Figure 27. A diagram illustrating the editing scheme on exon 3 of the *klotho* gene of fetuses generated by SCNT using non-selected porcine fibroblasts transfected with Cas9-sgRNA RNPs.

Table 10. Rate of DNA editing on the *klotho* gene of fetuses recovered after 28 days post embryo transfer

	No. of cloned fetuses	Type of editing events detected by deep sequencing		
		Unedited	Monoallelic	Biallelic
Absorbed	9	5 (55.6%)	4 (44.4%)	0 (0.0%)
Living	7	4 (57.1%)	3 (42.9%)	0 (0.0%)

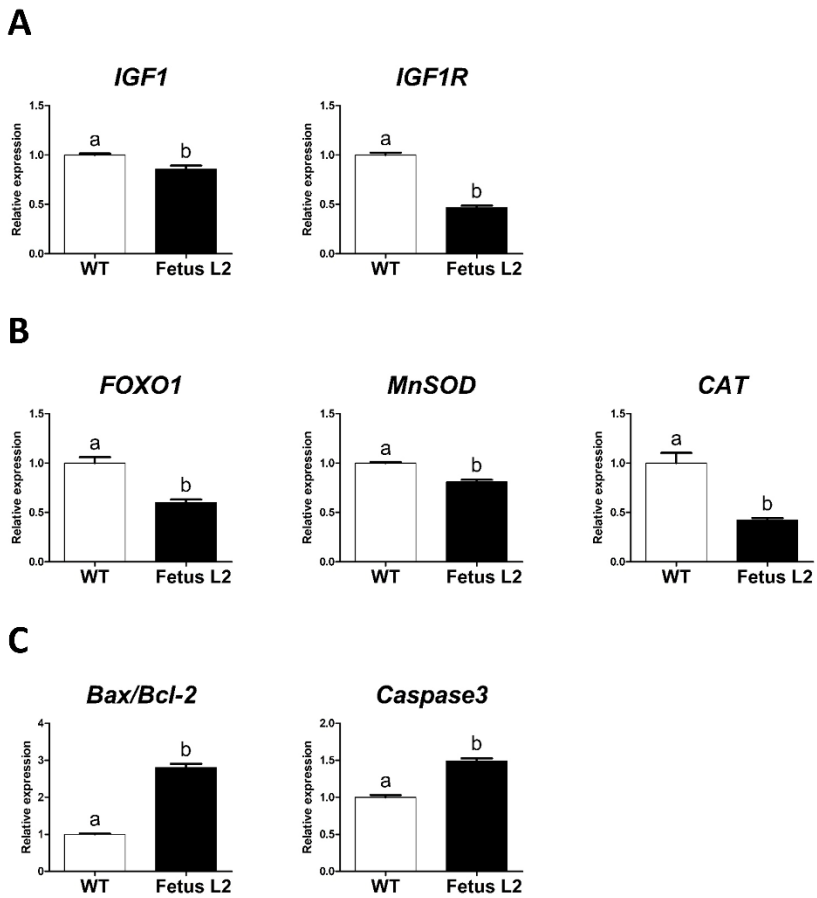


Figure 28. Expression of genes related to aging and apoptosis in *klotho* monoallelic knockout fetal fibroblasts. (A) IGF1 signaling genes (*IGF1* and *IGF1R*). (B) *FOXO1* and antioxidant genes (*MnSOD* and *CAT*). (C) Apoptosis-related genes (*Bax*/*Bcl2* ratio and *Caspase3*). Data are shown as the means ± SEM. Within each category, groups marked with different letters are significantly different ($P < 0.05$). The experiment was replicated at least three times. WT, wild type; Fetus L2, living fetus 2 (WT/-17bp,+12bp).

Table 11. Comparison of preimplantation development of the SCNT embryos derived from wild type and *klotho* monoallelic knockout porcine fibroblasts

Donor cell	No. of embryos cultured*	No. of embryos developed to (%)	
		≥ 2-cells*	Blastocyst**
WT	213	177 (82.8 ± 6.8)	32 (15.2 ± 5.0)
Fetus L2	220	189 (85.5 ± 4.7)	33 (14.3 ± 4.1)

Values with different superscript letters within a column are significantly different ($P < 0.05$). Experiment was replicated seven times. The data represent means ± SEM. WT, wild type; Fetus L2, living fetus 2 (WT/-17bp,+12bp).

* Percentage of the number of cultured embryos undergoing cleavage.

** Percentage of the number of cultured embryos developed into blastocysts.

Table 12. Results of transfer of SCNT embryos cloned from *klotho* monoallelic knockout porcine fetal fibroblasts

Recipient	Cell line	Type (genotype)	No. of transferred cloned embryos	Pregnancy	Remark
1	Fetus L3	monoallelic[a]	205	-	-
2			240	-	-
3			246	-	-
1	Fetus L2	monoallelic[b]	247	-	-
2			276	+	Abortion
3			212	+	Abortion
4			176	+	Recovery of fetuses and placentas
5			204	+	Recovery of fetuses and placentas
6			104	+	Abortion
7			92	+	Abortion
8			86	+	Abortion

[a] (WT/-1bp)

[b] (WT/-17bp,+12bp)

4. Discussion

Although recent studies have been conducted to investigate the functions of the *klotho* gene in mice, there is growing evidence that animal models other than rodents are required for biomedical applications [237, 238], due to inappropriate mimicking of some human genetic diseases [239]. Pigs are useful as animal models for human disease because these two species have many similarities in genetic background [240], anatomy and physiology [219].

Firstly, to establish *klotho* knockout fetal cell lines, I investigated embryo development and genome editing efficiency after SCNT using non-selected donor cells transfected with *klotho* targeting Cas9-sgRNA RNPs. Although a significant difference was observed in the fusion rates between non-transfected and transfected groups (95.5% vs. 89.4%), blastocyst formation rates between the groups were not significantly different. Blastocysts cloned from transfected cells showed 65.0% (13/20) modification rates in the T7E1 assay. To confirm the modifications, deep sequencing analysis was conducted and the results showed that among thirteen blastocysts, eight blastocysts contained monoallelic modifications (40.0%) and five blastocysts contained biallelic modifications (25.0%).

Based on high modification rates in preimplantation embryos, SCNT embryos cloned from non-selected donor cells, transfected with *klotho* targeting Cas9-sgRNA RNPs was transferred to five recipients. One of them became pregnant and sixteen cloned fetuses were recovered 28 days post transfer. Among the

sixteen fetuses, nine were absorbed and seven were living. Fetal fibroblasts cell lines were established from seven living fetuses by primary culture. Deep sequencing analysis revealed that three (42.9%) of seven living fetuses contained monoallelic modifications. Therefore, I established three *klotho* monoallelic knockout porcine fetal fibroblasts cell lines.

The *klotho* gene is considered to be an aging-suppressor gene. Although the mechanism of the anti-aging action of klotho is not fully elucidated, one solid hypothesis is that the ability of klotho to inhibit IGF1 signaling contribute to the anti-aging properties of klotho. The *klotho* overexpression in transgenic mice resulted in suppression of oxidative stress together with moderately inhibited IGF1 signaling [241]. Inhibition of IGF1 signaling by klotho can activate FOXO1 repressed by IGF1 signaling under normal conditions, and activated FOXO1 triggers activation of the antioxidant genes, including *CAT* and *MnSOD* [242], potentially contributing to anti-aging characteristics.

In order to investigate the properties of *klotho* monoallelic knockout fetal fibroblasts at the molecular level, the expression level of several genes related to aging and apoptosis was analyzed using wild type and Fetus L2 cells (WT/-17bp,+12bp). Fetus L2 had a significantly decreased expression level of *IGF1* and *IGF1R*. This result was consistent with a previous study that showed significantly decreased expression of *IGF1* and *IGF1R* mRNA in *klotho* biallelic knockout mice compared to wild type [243]. Activated IGF1 signaling by *klotho* monoallelic knockout may decrease expression of *IGF1* and *IGF1R* to inhibit excessive activation.

Subsequently, Fetus L2 also had significantly lower *FOXO1* and its downstream target genes with antioxidant function (*MnSOD* and *CAT*) than the wild type. Based on these results, *klotho* seems to exert anti-aging properties through inhibition of IGF1 signaling. In addition, increased apoptosis was reflected in an increased *Bax*/*Bcl2* ratio, an indicator of apoptosis [244] and *Caspase3*, one of the proapoptotic genes [245]. Conclusively, *klotho* monoallelic knockout in fetal fibroblasts negatively changed the expression of apoptosis-related genes.

Nextly, I investigated preimplantation development of SCNT embryos cloned from *klotho* monoallelic knockout porcine fibroblasts (Fetus L2). No significant difference was observed in cleavage rates and blastocysts formation rates between cloned embryos derived from wild type and Fetus L2 fibroblasts. These results indicated that *klotho* monoallelic knockout embryos were developed normally *in vitro*. Based on normal embryo development during preimplantation development, SCNT embryos cloned from *klotho* monoallelic knockout porcine fibroblasts (Fetus L2 and L3) were transferred to eleven synchronized recipients. Seven recipients (63.6%) became pregnant.

In conclusion, I established *klotho* monoallelic knockout fetal fibroblast cell lines that may be useful cell sources for SCNT procedures to generate *klotho* knockout pigs. In addition, I found that *klotho* monoallelic knockout downregulates antioxidant genes (*MnSOD* and *CAT*), which are downstream target genes of *FOXO1*. This phenomenon seems to be related to inhibition of FOXO1 through activation of IGF1 signaling by reduced klotho and can explain

the anti-aging properties of klotho. Collectively, I may predict that *klotho* knockout pigs will exhibit a shortened life span with altered IGF1 signaling, FOXO1 and subsequent antioxidant gene expression and will be a good model for studying aging of humans.

Chapter II. Production of cloned embryos using *klotho*-knockout porcine fibroblasts inducibly expressing a human *klotho* gene

1. Introduction

A defect in *klotho* gene expression in a mouse strain causes extremely shortened life span with multiple aging-like phenotypes similar to human premature-aging syndromes [141]. Mice with loss of klotho function (KL-/- mice) develop normally up to 3 weeks of age. Thereafter, they begin to manifest multiple aging-like phenotypes including growth retardation, infertility, arteriosclerosis, ectopic calcification in various soft tissues, osteoporosis, skin atrophy, muscle atrophy, pulmonary emphysema and finally premature death at 2 months of age.

In contrast, overexpression of *klotho* in mice prolongs life span by 20% [142]. Moreover, inducible *klotho* gene expression in klotho-deficient mice ($KL^{-/-}$ mice) rescues all the aging-like phenotypes generated before induction of the exogenous *klotho* gene [143]. In addition, expression of exogenously introduced *klotho* gene in limited tissues can rescue systemic $KL^{-/-}$ phenotypes [141] and parabiosis between $KL^{+/+}$ and $KL^{+/-}$ mice improved the impaired nitric oxide production seen in vascular endothelial cells of $KL^{+/-}$ mice [246]. In this regard, the *klotho* gene is considered to be one of the aging-suppressor genes that prolongs life span when overexpressed.

As limited information is available on the functions of the *klotho* gene in large animals such as pigs which are widely used as models for studying human disease

due to their anatomical and physiological similarities to humans [228], I recently established *klotho* monoallelic knockout porcine fetal fibroblasts. When I design an inducible *klotho* gene expression system in klotho-deficient pigs, the sequence of porcine *klotho* gene was not fully disclosed. The aim of this study, therefore, was to generate *klotho* monoallelic knockout porcine fibroblasts inducibly expressing a human *klotho* (*hKlotho*) gene and preimplantation embryos cloned from this cell line in order to produce a more suitable animal model for elucidating the role of this gene in aging.

2. Materials and methods

2.1. Animals

The animals used in this study were maintained by the R&F farm (Boryeong, Korea) and the research farm of Gyeonggi Livestock and Veterinary Service (Osan, Korea). All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of Seoul National University (SNU-160613-16) in accordance with the Guide for the Care and Use of Laboratory Animals of Seoul National University.

2.2. Generation of the *hKlotho* overexpression cell line

hKlotho (from Addgene, http://www.addgene.org, Plasmid # 17712) was linked to an *mCherry* with F2A sequences by PCR amplification, and inserted into the piggybac transposon expression vector (pB-CA, addgene Plasmid # 20960) using the In-Fusion cloning kit (Clontech, CA, USA). After the transfection of 1,000 ng of pB-CA-*hKlotho*-F2A-*mCherry* and transposase (named pCy43, provided by The Sanger Institute, Hinxton, UK) into 5×10^5 of porcine fibroblasts using nucleofection (Neon, Invitrogen) with a 1,400 v, 20 ms pulse width, and pulse number 2 setting, the mCherry-positive cells were mechanically isolated using micromanipulation and subcultured. This cell line was named CA-*hKlotho*.

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2.3. Generation of tetracycline (Tet)-inducible *hKlotho* overexpression cell line

hKlotho was linked to an *mCherry* with F2A sequences by PCR amplification, and inserted into the piggybac transposon expression vector (pB-TET, addgene Plasmid #20909) using the In-Fusion cloning kit (Clontech). After the transfection of 1,000 ng of pB-Tet-*hKlotho*-F2A-*mCherry*, transposase (named pCy43, provided by The Sanger Institute), and pB-CAG-rtTA into 5×10^5 of porcine fibroblasts via nucleofection (Neon, Invitrogen) with a 1400 v, 20 ms pulse width, and pulse number 2 setting, 1,000 μg/ml neomycin (G418; Gibco) was used for 7 days to isolate the transfected cells and then growing cells resistant to neomycin were subcultured. This cell line was named Tet-*hKlotho* (+) when treated with doxycycline and Tet-*hKlotho* (-) when left untreated.

2.4. PCR

Genomic DNA was extracted with the G-spin Genomic DNA Extraction Kit (iNtRON Biotechnology, Gyeonggi-do, Korea) according to the manufacturer's protocol. For the amplification of target genes, PCR was conducted using Maxime PCR PreMix (i-StarTaq, iNtRON). The PCR reactions were denatured at 95 ˚C for 20 s, annealed at 60 ˚C for 10 s and extended at 72 ˚C for 30 s. A primer set for *hKlotho* (forward primer: 5'-TGGACCCACCTTGAGTTTTC-3'and reverse primer: 5'-GGAGGGAAGCCATTTTTCTC -3', 411bp) was used.

2.5. RT-PCR

To detect transcripts of *hKlotho*, the total RNAs were extracted with the easy-spin Total RNA Extraction Kit (iNtRON) and added to the Maxime RT Premix

kit (oligo (dT) primer; iNtRON) for the synthesis of cDNAs. Plasmid vector (pB-CA-*hKlotho*-F2A-*mCherry* or pB-Tet-*hKlotho*-F2A-*mCherry*) was used as a positive control for *hKlotho*. Primer sets for transcript of *hKlotho* (forward primer: 5'-TGGACCCACCTTGAGTTTTC-3' and reverse primer: 5'-GGAGGGAAGCCATTTTTCTC -3', 411bp) and *β-actin* (forward primer: 5'-GTGGACATCAGGAAGGACCTCTA-3'and reverse primer: 5'-ATGATCTTGATCTTCATGGTGCT -3', 137bp) were used.

2.6. Somatic cell nuclear transfer

Procedures for SCNT were described in general methodology. The combination of 2 μM resveratrol and 10^{-9} M melatonin was supplemented to the IVM medium during the entire maturation period, when we performed SCNT for embryo transfer.

2.7. Embryo transfer and pregnancy test

Procedures for embryo transfer and pregnancy test were described in general methodology.

2.8. Statistical analysis

Procedures for statistical analysis were described in general methodology.

3. Results

3.1. Establishment of CA-*hKlotho* and Tet-*hKlotho* cell lines

The *hKlotho*-F2A-*mCherry* transgene was constructed under the control of the CMV enhancer/chicken β-actin promoter (CAG) (Fig. 29A). The constructed transgene was introduced into porcine fetal fibroblast cells using a nucleofection gene delivery system. After transfection, the mCherry-positive cells were mechanically isolated and subcultured. Establishment of the CA-*hKlotho* cell line was further confirmed by mCherry expression observed with fluorescence microscopy (Fig. 30B and 30B').

To establish the Tet-inducible *hKlotho* cell line (Tet-*hKlotho*), porcine fetal fibroblasts were transfected with a vector containing *hKlotho*, whose expression was controlled by doxycycline. (Fig. 29B). The transfected fibroblasts were selected with medium containing neomycin, which was further confirmed by mCherry expression with (Fig. 30C and 30C') or without doxycycline (Fig. 30D and 30D').

Integration and expression of the *hKlotho* gene were observed by genomic DNA PCR (Fig. 31A) and RT-PCR (Fig. 31B) in wild type, CA-*hKlotho*, Tet-*hKlotho* (+) and Tet-*hKlotho* (-) cells, respectively. PCR and RT-PCR data indicated that the *hKlotho* gene was integrated into porcine fetal fibroblasts. I compared transcript expression levels of *hKlotho* measured by RT-PCR (Fig. 31C) and the CA-*hKlotho* group displayed the highest *hKlotho* expression compared to the other groups. In the Tet-*hKlotho* (+) group treated with doxycycline,

expression of *hKlotho* was significantly increased compared to Tet-*hKlotho* (-) cells.

3.2. Preimplantation development of cloned embryos derived from CA-*hKlotho* and Tet-*hKlotho* (+) cell lines

Development rates were evaluated in three groups: cloned embryos derived from wild type, CA-*hKlotho* and Tet-*hKlotho* (+) cells. There was no significant difference in cleavage rates (78.5%, 80.5% and 77.3%, respectively). However, cloned embryos derived from *hKlotho* overexpression cells (CA-*hKlotho* and Tet-*hKlotho* (+) cells) significantly increased blastocyst formation rates (21.5% and 20.2%) compared to wild type (8.4%) (Table 13).

3.3. Establishment of *klotho* monoallelic knockout porcine fibroblasts inducibly expressing a *hKlotho*

To establish the *klotho* monoallelic knockout porcine fibroblasts inducibly expressing a *hKlotho*, Fetus L2 fibroblasts (*klotho* monoallelic knockout) were transfected with a Tet-inducible vector (pB-Tet-*hKlotho*-F2A-*mCherry*) (Fig. 29B). The transfected fibroblasts were selected using neomycin for 7 days. These selected fibroblasts were confirmed by mCherry expression with (Fig. 32B and 32B') or without doxycycline (Fig. 32C and 32C').

Integration and expression of the *hKlotho* gene were verified by genomic DNA PCR (Fig. 33A) and RT-PCR (Fig. 33B) in Fetus L2, Fetus L2/Tet-*hKlotho* (+) and Fetus L2/Tet-*hKlotho* (-) cells, respectively. The results showed that the

hKlotho gene was integrated into *klotho* monoallelic knockout porcine fibroblasts. Furthermore, transcript expression levels of *hKlotho* was measured by RT-PCR (Fig. 33C). When Fetus L2/Tet-*hKlotho* (+) cells were treated with doxycycline, expression of *hKlotho* was significantly increased compared to Fetus L2/Tet-*hKlotho* (-) cells.

3.4. Preimplantation development of cloned embryos derived from Fetus L2 and Fetus L2/Tet-*hKlotho* (+) cell lines

No significant difference was observed in cleavage rates between cloned embryos derived from Fetus L2 and Fetus L2/Tet-*hKlotho* (+) cells (Table 14). However, cloned embryos derived from Fetus L2/Tet-*hKlotho* (+) cells showed significantly increased blastocyst formation rates compared to cloned embryos derived from Fetus L2 (19.7% vs. 12.7%).

3.5. Results of transfer of SCNT embryos cloned from *klotho* monoallelic knockout porcine fetal fibroblasts expressing inducibly a human *klotho* gene.

As shown in Table 15, SCNT embryos cloned from *klotho* monoallelic knockout porcine fibroblasts with or without inducibly expressing a *hKlotho* (284 and 416, respectively) were transferred to three synchronized recipients. All recipients (100.0%) became pregnant. However, all of them were aborted.

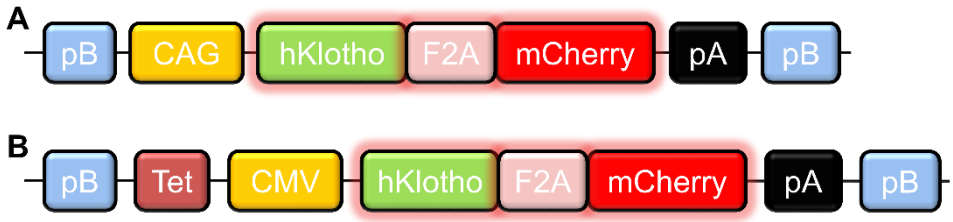

Figure 29. A schematic diagram of constructs used in the study. *hKlotho* was linked to an *mCherry* with F2A sequences by PCR amplification, and inserted into the piggybac transposon expression vector (pB-CA or pB-Tet) by In-Fusion cloning. (A) pB-CA-*hKlotho*-F2A-*mCherry*. In this vector, *hKlotho* expression is under the control of the CAG promoter. (B) pB-Tet-*hKlotho*-F2A-*mCherry*. Expression of the *hKlotho* is controlled by doxycycline-dependent promoter. CAG, CMV enhancer/chicken b-actin; *hKlotho*, human *klotho*; PCR, polymerase chain reaction.

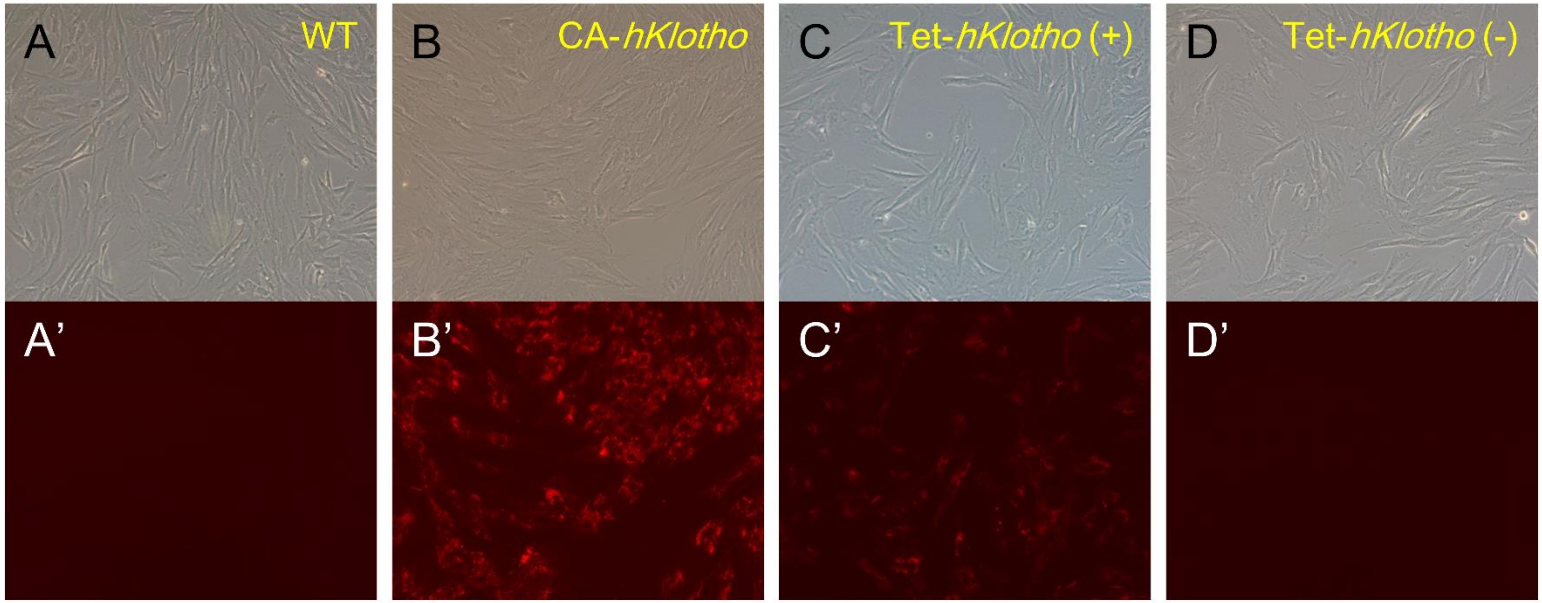

Figure 30. *hKlotho*-F2A-*mCherry* expression in CA-*hKlotho*, Tet-*hKlotho* (+), and Tet-*hKlotho* (-) cell lines. (A, A') Wild type porcine fibroblast cell line. No expression of mCherry was detected. (B, B') CA-*hKlotho* cell line. The mCherry expression was confirmed under UV. (C, C') Tet-*hKlotho* (+); Tet-*hKlotho* cell line was cultured in the presence of doxycycline (2 μg/mL). The mCherry expression was induced by doxycycline. (D, D') Tet-*hKlotho* (-); Tet-*hKlotho* cell line was cultured in the absence of doxycycline. No expression of mCherry was detected.

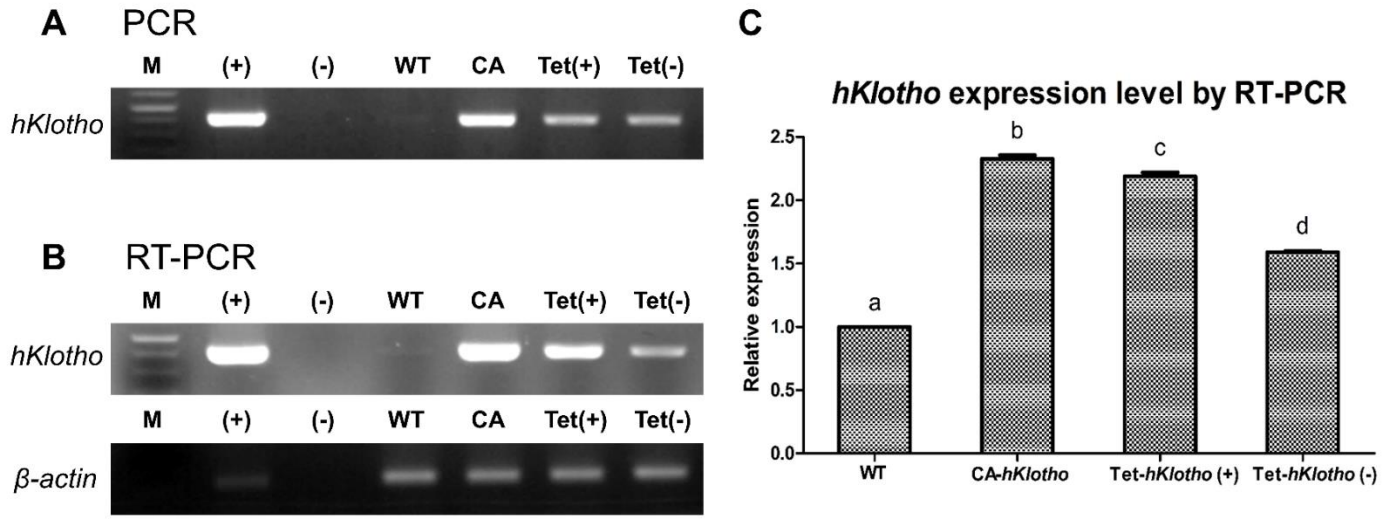

Figure 31. PCR and RT-PCR results for transfected porcine fibroblasts. (A) Insertion of *hKlotho* in CA-*hKlotho*, Tet-*hKlotho* (+), and Tet-*hKlotho* (-) cell lines identified by PCR. (B) Transcription of *hKlotho* in CA-*hKlotho*, Tet-*hKlotho* (+), and Tet-*hKlotho* (-) cell lines was also confirmed by RT-PCR. M, Marker; (+), positive control (plasmid vector; pB-CA-*hKlotho*-F2A-*mCherry*); (-), negative control; WT, nontransfected wild type porcine fibroblast cell line; CA, CA-*hKlotho* cell line; Tet(+), Tet-*hKlotho* (+) cell line; Tet(-), Tet-*hKlotho* (-) cell line. (C) Comparison of *hKlotho* expression level measured by RT-PCR. Within each endpoint, bars with different superscript letters (a–d) are significantly different ($P < 0.05$). RT-PCR, reverse transcription-PCR.

Table 13. Comparison of preimplantation development of the SCNT embryos derived from wild type, CA-*hKlotho* and Tet-*hKlotho* (+) cell lines

Donor cell	No. of embryos cultured*	No. of embryos developed to (%)	
		≥ 2-cells*	Blastocyst**
WT	97	77 (78.5 ± 5.9)	9 (8.4 ± 1.5)[a]
CA-*hKlotho*	100	82 (80.5 ± 4.6)	24 (21.5 ± 3.9)[b]
Tet-*hKlotho* (+)	102	80 (77.3 ± 2.8)	23 (20.2 ± 4.0)[b]

Values with different superscript letters within a column are significantly different ($P < 0.05$). Experiment was replicated four times. The data represent means ± SEM. WT, wild type; CA-*hKlotho*, porcine fibroblasts transfected with pB-CAG-*hKlotho*-F2A-*mCherry*; Tet-*hKlotho* (+), porcine fibroblasts transfected with pB-Tet-*hKlotho*-F2A-*mCherry*, which was treated with doxycycline.

* Percentage of the number of cultured embryos undergoing cleavage.

** Percentage of the number of cultured embryos developed into blastocysts.

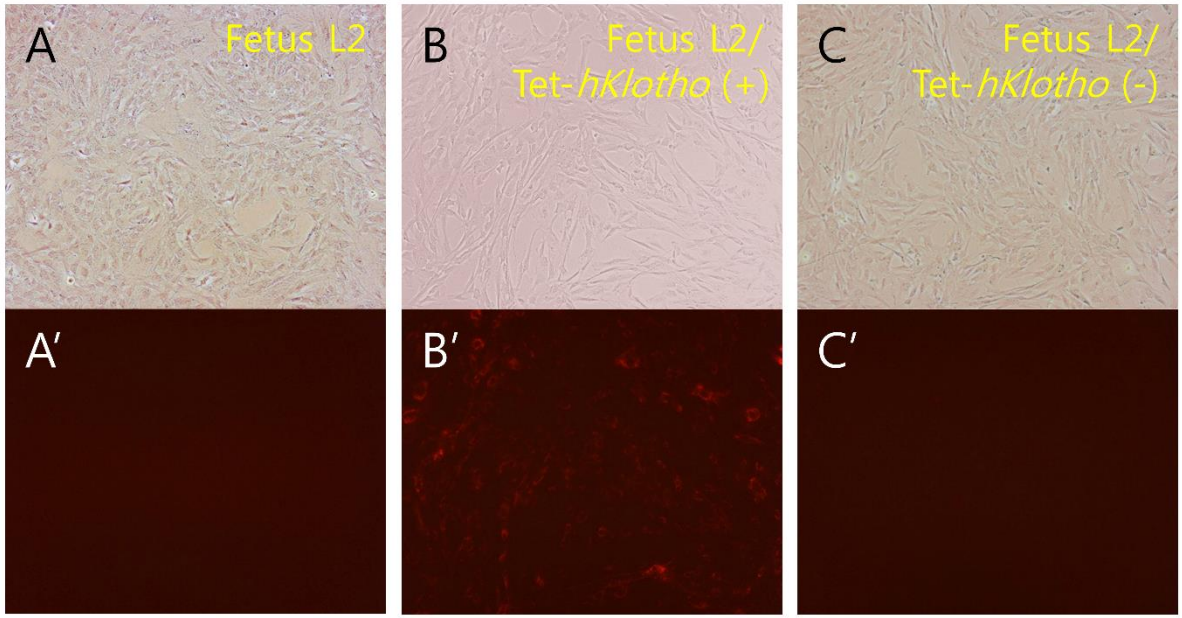

Figure 32. *hKlotho*-F2A-*mCherry* expression in Fetus L2/Tet-*hKlotho* (+) and Fetus L2/Tet-*hKlotho* (-) cell lines. (A, A') Fetus L2 cell line (WT/-17bp,+12bp). No expression of mCherry was detected. (B, B') Fetus L2/Tet-*hKlotho* (+); Fetus L2/Tet-*hKlotho* cell line was cultured in the presence of doxycycline (2 μg/mL). The mCherry expression was induced by doxycycline. (C, C') Fetus L2/Tet-*hKlotho* (-); Fetus L2/Tet-*hKlotho* cell line was cultured in the absence of doxycycline. No expression of mCherry was detected.

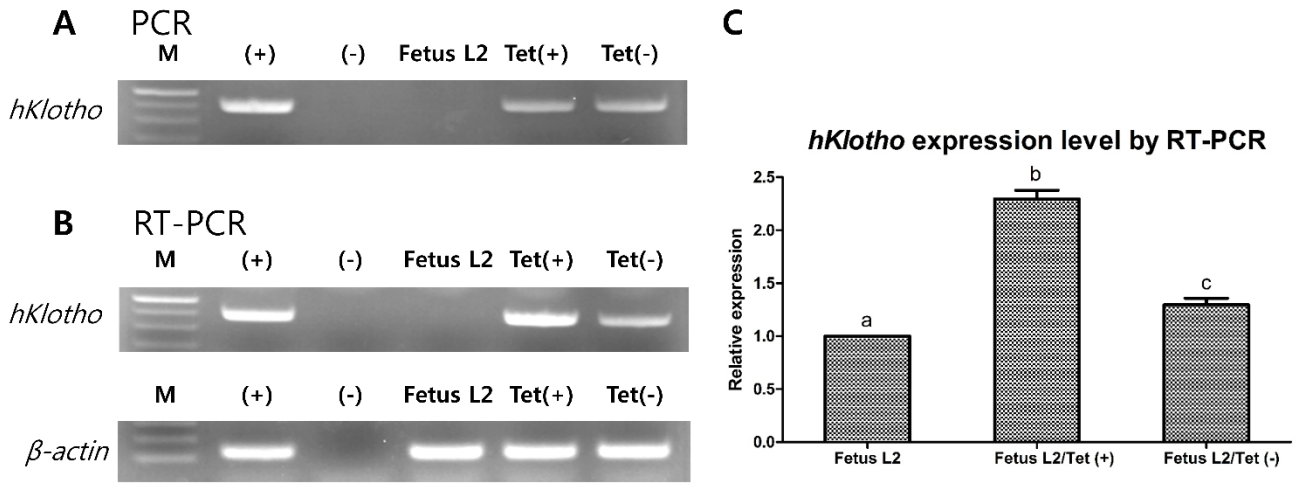

Figure 33. PCR and RT-PCR results for transfected *klotho* monoallelic knockout porcine fibroblasts. (A) Insertion of *hKlotho* in Fetus L2/Tet-*hKlotho* (+) and Fetus L2/Tet-*hKlotho* (-) cell lines identified by PCR. (B) Transcription of *hKlotho* in Fetus L2/Tet-*hKlotho* (+) and Fetus L2/Tet-*hKlotho* (-) cell lines was also confirmed by RT-PCR. M, Marker; (+), positive control (plasmid vector; pB-CA-*hKlotho*-F2A-*mCherry*); (-), negative control; WT, nontransfected wild type porcine fibroblast cell line; Tet(+), Fetus L2/Tet-*hKlotho* (+) cell line; Tet(-), Fetus L2/Tet-*hKlotho* (-) cell line. (C) Comparison of *hKlotho* expression level measured by RT-PCR. Within each endpoint, bars with different superscript letters (a–c) are significantly different ($P < 0.05$). RT-PCR, reverse transcription-PCR.

Table 14. Comparison of preimplantation development of the SCNT embryos derived from *klotho* monoallelic knockout porcine fibroblasts with or without inducibly expressing a human *klotho* gene

Donor cell	No. of embryos cultured*	No. of embryos developed to (%)	
		≥ 2-cells*	Blastocyst**
Fetus L2	109	91 (83.2 ± 6.3)	14 (12.7 ± 2.3)[a]
Fetus L2/Tet-*hKlotho* (+)	116	99 (85.3 ± 1.9)	22 (19.1 ± 2.8)[b]

Values with different superscript letters within a column are significantly different ($P < 0.05$). Experiment was replicated five times. The data represent means ± SEM. Fetus L2, living fetus 2 fibroblasts (WT/-17bp,+12bp); Fetus L2/Tet-*hKlotho* (+), living fetus 2 fibroblasts transfected with pB-Tet-*hKlotho*-F2A-*mCherry*, which was treated with doxycycline.

* Percentage of the number of cultured embryos undergoing cleavage.

** Percentage of the number of cultured embryos developed into blastocysts.

Table 15. Results of transfer of SCNT embryos cloned from *klotho* monoallelic knockout porcine fetal fibroblasts expressing inducibly a human *klotho* gene

Recipient	Cell line	Type (genotype)	No. of transferred cloned embryos	Pregnancy	Remark
1	Fetus L2 + Fetus L2/Tet-*hKlotho*	monoallelic[a] + monoallelic[a]/Tet-*hKlotho*	289 (173+116)	+	Abortion
2			129 (65+64)	+	Abortion
3			282 (178+104)	+	Abortion

[a] (WT/-17bp,+12bp)

4. Discussion

When *klotho* expression was induced in *klotho* biallelic knockout mice carrying an exogenous *klotho* gene, in which *klotho* expression was dependent on zinc water feeding, many advanced aging-like phenotypes were restored to normal [143]. In addition, parabiosis between wild type and *klotho* monoallelic knockout mice resulted in restoration of endothelial function in *klotho* monoallelic knockout mice, which showed impaired nitric oxide production in vascular endothelial cells [246]. In this study, I established *klotho* monoallelic knockout porcine fetal fibroblasts inducibly expressing a *hKlotho*. The *hKlotho* was selected instead of porcine *klotho*, because the sequence of porcine *klotho* gene was not fully disclosed, when this study was designed.

Firstly, I established two vectors and cell lines: One is the CA-*hKlotho* cell line, which overexpresses *hKlotho* under the control of the CAG promoter (pB-CA-*hKlotho*-F2A-*mCherry*) and the other is the Tet-*hKlotho*, which overexpresses *hKlotho* under doxycycline-dependent promoter (pB-Tet-*hKlotho*-F2A-*mCherry*). Although doxycycline was used, the Tet-*hKlotho* (+) cell line expressed significantly lower *hKlotho* than CA-*hKlotho*. This was confirmed by observation of mCherry expression with a fluorescent microscope and comparison of transcript levels of *hKlotho* by RT-PCR. The reason why mCherry expression can confirm the expression of *hKlotho* is because *hKlotho* is directly connected to *mCherry* by the F2A sequence and they should therefore be expressed at the same level.

One disadvantage of tetracycline-inducible systems is their well described leakiness [247], which may be attributed to chromosomal integration, false promoters, or cryptic initiation signals under non-induced conditions [248, 249]. Considering that the level of *hKlotho* in Tet-*hKlotho* (-) cell line was significantly higher than the wild type group, our inducible system also seems to be somewhat leaky. The *hKlotho* overexpression cell lines were employed in porcine SCNT to compare embryo development. Embryos cloned from CA-*hKlotho* and Tet-*hKlotho* (+) cell lines had significantly higher blastocyst formation rates than the wild type.

After confirmation of the expression of *hKlotho* by vectors, I established *klotho* monoallelic knockout porcine fibroblasts inducibly expressing a *hKlotho* (Fetus L2/Tet-*hKlotho* (+)), using the Tet inducible vector (pB-CA-*hKlotho*-F2A-*mCherry*). This cell line was also confirmed by PCR, RT-PCR and mCherry expression and employed in porcine SCNT to compare embryo development. SCNT embryos cloned from Fetus L2/Tet-*hKlotho* (+) showed significantly higher blastocyst formation rates compared to Fetus L2. Based on the preimplantation embryo development results, there is a possibility that *hKlotho* expression influences blastocyst formation rates. As the epigenetic condition of donor cells could affect cloning efficiency [250], this might be due to epigenetic modification by the *klotho* gene [251]. However, further studies are required to elucidate underlying mechanism by which *hKlotho* expression affects blastocyst formation rates.

To produce *klotho* monoallelic knockout pigs inducibly expressing a *hKlotho*, embryo transfer was conducted. A total of 700 SCNT embryos cloned from *klotho* monoallelic knockout porcine fibroblasts with or without inducibly expressing a *hKlotho* were transferred to three synchronized recipients and all of recipients became pregnant. However, they all were aborted.

In conclusion, I established *klotho* monoallelic knockout porcine fetal fibroblasts inducibly expressing *hKlotho* that may be useful cell sources for SCNT procedures to generate a more appropriate model for studying aging of humans.

PART V

FINAL CONCLUSION

This thesis was conducted to establish Shh signaling-induced *in vitro* oocyte maturation system and to apply this system to *klotho*-knockout pig production.

Firstly, the relationship between the beneficial effects of resveratrol or melatonin on porcine IVM was demonstrated. Furthermore, the synergistic effects of the combination of resveratrol and melatonin on porcine oocyte nuclear maturation and total cell numbers of PA blastocysts was demonstrated. Finally, the combination improved porcine SCNT efficiency.

Secondly, *klotho* monoallelic knockout fetal cell lines with or without inducibly expressing a human *klotho* gene were firstly established in the pig. The *klotho* monoallelic knockout fetal cell lines were generated by recovery of fetuses cloned via SCNT using non-selected porcine fibroblasts transfected with Cas9-sgRNA RNPs, targeting exon 3 of the porcine *klotho* locus. The *klotho* monoallelic knockout fetal cell line inducibly expressing a human *klotho* gene was established by transfection of a tetracycline inducible vector (Tet-*hKlotho*-F2A-*mCherry*). Lastly, using oocytes matured *in vitro* with the combination of resveratrol and melatonin and these *klotho*-knockout cell lines as nuclear donors, cloned embryos were produced via SCNT and transferred to recipients to produce *klotho*-knockout pigs.

In this thesis, I demonstrated the relationship between the beneficial effects of resveratrol or melatonin on porcine IVM and Shh signaling and their synergistic effects on porcine IVM and subsequent embryo development after PA and SCNT. Moreover, I demonstrated an efficient method for establishment of *klotho*-knockout fetal cell lines by recovering fetuses cloned via SCNT using non-

selected fibroblasts transfected with Cas9-sgRNA RNPs. Furthermore, the *klotho* monoallelic knockout embryos were developed until day 7 *in vitro* without showing any abnormalities and transferred to recipients to produce *klotho*-knockout pigs. Based on the results of the present study, Shh signaling-induced IVM system will be useful for generating pig models for studying human diseases.

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국문초록

Sonic hedgehog signaling 이 촉진된 체외난자성숙체계의 확립 및 *klotho* 유전자 제거 돼지 생산을 위한 이의 적용

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돼지는 해부학 및 생리학적으로 인간과 매우 유사하기 때문에, 의생명과학연구에서 모델동물로써 다양하게 사용되어 왔다. 특히, 유전자 변형 돼지는 특정 유전자를 제거함으로써, 인간의 질병을 연구하거나 이종장기 이식을 위하여 모델동물로 사용될 수 있다. 착상전 돼지 배아의 체외 생산법은 특별한 목적을 갖는 동물을 생산하는 것을 가능하게 했으며, 그 중 체세포복제는 유전자 변형 돼지를 만드는 가장 효율적인 방법 중 하나이다. 하지만 체세포복제 과정에서 필수 요건인, 체외에서 성숙된 난자의 발달능이 아직 낮은 수준이다. 돼지 난자의

체외성숙을 향상시키기 위해 배양 시 특정화합물의 효과를 보는 많은 연구가 있었지만, 난자 성숙의 생리 과정에 대한 이해와 이 화합물들이 돼지 난구세포-난자 복합체에 미치는 영향에 대한 기저 원리에 대해서는 더 연구가 필요하다.

난포 내에서 난자가 성숙하기 위해서는 과립막세포, 난구세포 들과 Shh 같은 signaling 을 통해 서로의 증식과 분화를 조절하는 communication 을 한다. 최근에는, 돼지 난소의 과립막세포와 난구세포층에서 Shh signaling 의 타겟들 (Ptch, Smo 및 Gli1)이 발견되었다. 이러한 배경에 의해, Shh 는 난구세포의 확장과 난자 성숙에 영향을 주는 중요한 signaling 으로 고려된다. 따라서, 이 연구에서는, Shh signaling 이 촉진된 체외난자성숙체계를 확립하고, *klotho* 유전자 제거 돼지 생산을 위한 이의 적용가능성에 대하여 연구하였다.

첫 번째로, resveratrol 및 melatonin 이 체외난자성숙체계에 미치는 영향과 Shh signaling 과의 관계에 대하여 알아보았다. Resveratrol 과 melatonin 이 돼지 난구세포-난자 복합체에 직접적으로 미치는 근본적인 기전을 밝히기 위하여, 난구세포의 확장, 난자의 핵성숙, 추후 발달 및 Shh signaling 관련 유전자 및 단백질의 발현 등을 분석하였다. Shh signaling 억제제인 cyclopamine 을 동시에 처리시 resveratrol 또는 melatonin 만 처리하였을 때

나타났던 난구세포의 확장 증가, cumulus cell 에서의 Shh signaling 유전자 및 단백질의 발현 증가 및 추후 배아 발달의 증가와 같은 효과는 보이지 않았다. 따라서, resveratrol 또는 melatonin 이 Shh signaling 을 통해 돼지 난자의 체외성숙 및 추후 배아 발달 향상시킨다는 것을 확인하였다. 다음 연구로, resveratrol 과 melatonin 의 조합 처리가 돼지 난자의 체외성숙에 미칠 수 있는 시너지 효과에 대하여 분석하였다. 그 결과, resveratrol 과 melatonin 의 조합 처리군에서 난자의 핵성숙과 단위발생 배반포의 총 세포수 향상에 시너지효과를 보였으며, 최종적으로 체세포복제 배아의 배반포 형성율과 총 세포수 증가를 확인하였다.

두 번째로, 이러한 체외난자성숙체계를 *klotho* 유전자 제거 돼지 생산에 적용하였다. *Klotho* 유전자는 수명을 조절하는 노화 억제 유전자 중 하나이다. 돼지와 같은 대형동물에서 *klotho* 유전자의 기능에 대한 정보가 부족하기 때문에, *klotho* 유전자 3 번 exon 을 타겟팅하는 Cas9-sgRNA 리보핵단백질을 돼지 세포에 electroporation 방법으로 주입한 후, 선별 없이 체세포복제에 사용한 후 이식하여 28 일 후 태아를 회수하여 *klotho* monoallelic 제거 돼지 세포주들을 확립하였다. 이 *klotho* 제거 세포주들을 공여세포로써 사용하여 *klotho* 제거 복제 배아를 제작하였고, *klotho* 유전자 제거 돼지 생산을 위하여 11 마리의 대리모에 이식하였다. 대리모 11 마리 중

7 마리 (63.6%)가 초기 임신으로 확인되었다. 다음으로, pB-Tet-*hKlotho*-F2A-*mCherry* 벡터를 이용하여 인간 *klotho* 유전자를 유도발현하는 *klotho* monoallelic 제거 세포주를 확립하였고, 이 세포주를 이용하여 복제한 복제배아를 3 마리의 대리모에 이식하였다. 대리모 3 마리 모두 (100.0%) 초기 임신이 확인되었다.

결론적으로, 돼지난자의 체외성숙시 resveratrol 처리 및 melatonin 의 처리는 Shh signaling 의 활성화를 통해 체외성숙을 향상시켰으며, 이들을 함께 처리하였을 때는 단독처리에 비해 돼지 난자의 체외성숙에 시너지효과가 있었다. 또한, 최종적으로 체세포복제 효율을 향상시켰다. 뿐만 아니라, 인간 *klotho* 유전자를 유도발현하거나 하지 않는 *klotho* monoallelic 제거 태아유래 섬유아세포 세포주를 돼지에서 최초로 확립하였다. Resveratrol 과 melatonin 의 조합에 의해 성숙된 난자를 공여난자로 사용 하고, *klotho* 유전자 제거 세포주들을 공여세포로 이용하여 체세포복제를 통해 복제배아를 제작하였으며, *klotho* 유전자 제거 돼지 생산을 위해 대리모에 이식하였다. 이식 28 일 후 초음파 진단을 통해 성공적인 착상이 이루어짐을 확인하였다. 본 연구를 통해 확립한 Shh signaling 이 촉진된 난자체외성숙체계는 인간질병연구를 위해 필수적인 질병모델로서 유전자변형 돼지를 생산하는데 효율적으로 적용이 가능함을 증명하였다.

주요어: 체외성숙, sonic hedgehog signaling, 체세포복제, *klotho*, 돼지

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